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The Carcinostatic Effect of Extracts Prepared from Cow Ovaries [1,2]

T. R. Tosteson, S. A. Ferguson, E. Davidson, *and* R. J. Rutman,[3] *Department of Zoology, University of Pennsylvania, Philadelphia, Pennsylvania*

It has been suggested that the rate of cell division, indeed cell division itself, may be due to a process dependent on the balance of heparin-like anticoagulant substances and their antagonists. The abnormal rate of division observed in malignant cells has been attributed to the lack or disfunction of one of the components of this regulatory balance (*1*).

This general concept is the basis of the work of which this paper constitutes a part. In the mammalian ovary there is a marked regulation of cell division. Aqueous extracts were made of cow ovaries and tested against the growth and lethality of an ascites tumor implanted in mice. The antimitotic action of such extracts on marine eggs has been reported previously (*2, 3*).

Animals inoculated with an ascites tumor were treated with extracts of cow ovaries. The growth of these tumors has been reported by others (*4*). The present work summarizes the carcinostatic effect of a variety of extracts prepared from cow ovaries, in terms of the survival of cancerous mice treated with such preparations.

Materials and Methods

A. Extraction Procedures

In the extractions outlined in table 1 the same general procedure was followed. Whole cow ovaries were obtained and cleaned. The connective tissue was trimmed away from each ovary and the resulting material ground in a meat grinder, and then weighed. The ground ovarian material was placed in the extraction medium for varying lengths of time as designated in table 1. In extraction procedures 1 to 3, antibiotics were added to the extraction media. After extraction, the preparation was cen-

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[2] This investigation was supported by research grant CY-2442 from the National Cancer Institute of the National Institutes of Health, Public Health Service, and by the American Cancer Society.

[3] The authors wish to express their appreciation and gratitude to Dr. L. V. Heilbrunn, of the Zoology Department of the University of Pennsylvania, for his constant aid and support of the work presented here, and to Dr. H. Borei for the use of his constant-temperature room, and to the Cross Brothers Meat Packing Company, of Philadelphia, Pennsylvania, for supplying the ovarian material for extraction.

trifuged in a refrigerated International Centrifuge for 30 minutes at 2000 r.p.m. The resulting supernatant extract was recentrifuged for 1 hour at 20,000 × g in a Spinco Centrifuge. The supernatant solution from this second centrifugation was placed in a cold room at 4° C. and fractionated by the addition of cold ethyl alcohol. The precipitates obtained from the alcholic solution were lyophilized and stored in a desiccator until used.

TABLE 1.—*Outline of extraction procedures*

Procedure and extract No.	Extraction medium	Extraction ratio (*ml.*:*gm.*)	Temperature	Extraction time (*hrs.*)	Fractionation
1	0.9 percent NaCl	2:1	4° C.	12–17	Cold alcohol precipitation
2	1 *M* NaCl	2:1	4° C.	12–17	Cold alcohol precipitation
3	Acetate *p*H 4.5 "acid"	2:1	4° C.	12–17	Cold alcohol precipitation.
4	0.5 *N* NaOH *p*H 12.0 "basic"	2:1	37° C.	14	Acidified *p*H 4 cold alcohol precipitation
5	"Phenolic"	5:4:1	25° C.	¾	Dialysis of phenolic layer (top) *vs.* distilled H_2O

Extraction procedure 5 was somewhat different from the previous extractions. One part ground ovarian material was placed in a double-layered solution of 5 parts saturated sodium acetate and 4 parts 90 percent phenol. Following the period of extraction, the phenolic (top) layer was removed and dialyzed against distilled H_2O. A precipitate formed within the dialysis sac. This precipitate was removed, washed with acetone, lyophilized, and subsequently tested.

B. Purification of Crude Fractions

The crude preparations obtained by extraction procedures 4 and 5 were further purified. The phenol remaining in the powdered phenolic-precipitate was removed by combining the precipitate with diethyl ether, 60 ml. per gm. of precipitate, and mixing in a Waring blendor. Fractions from both 4 and 5 were then treated in the following manner:

The powdered material was placed in a solution of 3 parts ethyl alcohol to 1 part ether (50 ml. of solution per gm. of extract) and kept at a temperature of 45° C. for 30 minutes. The mixture was centrifuged and the supernatant discarded. This procedure was repeated 3 times. Subsequently, the material was further treated by placing it in a solution of 1 *M* NaCl for 1 hour at 100° C. (50 ml. of solution per gm. of material). The mixture was centrifuged and the supernatant solution discarded.

This procedure was then repeated with 30 ml. of solution per gm. of material. Following the second 1 *M* NaCl wash, the mixture was centrifuged and the precipitate washed with water. The solid material remaining was then lyophilized. In the subsequent discussion this fraction is designated as the "protein" fraction. The above procedures were designed to rid the extract of loosely bound lipides and nucleic acids (*5*).

C. The Tumor and the Mice

The Ehrlich ascites EF hypodiploid tumor used in these studies was originally obtained in October, 1955, through the kindness of Dr. Creech, of the Institute for Cancer Research, Fox Chase, Pennsylvania. The tumor was carried in white Swiss mice by weekly serial intraperitoneal transfers of approximately 10×10^6 tumor cells in 0.2 ml. ascitic fluid. The white Swiss mice used in these studies were obtained from R. Detwiler, Cedars, Pennsylvania.

D. Experimental Procedure

Mice.—The tumor cells were inoculated intraperitoneally into the mice at a dose of 1.5×10^6 tumor cells in 0.2 ml. of fluid. The cells were allowed to multiply for 24 hours before treatment was begun. The treatment consisted of 5 daily intraperitoneal injections of the extract to be tested. The "crude" material was suspended in 0.9 percent NaCl at a concentration of 30 mg. of dry extract per ml. The more purified preparations were suspended in distilled water. Each mouse received a daily inoculation of 0.5 ml. or 15 mg. of dry material. A treated animal received a total of 75 mg. of dry material.

Two parameters of tumor growth which are used to measure the efficacy of a treatment are (*a*) the survival of the animal and (*b*) the multiplication of cells in the peritoneal cavity. In the work reported here, only one of these parameters will be discussed, that of survival of the animal. A study of the effect of ovarian extracts on the multiplication of tumor cells and the chemical composition of the active material is to be published separately. Mice inoculated with the tumor were observed for 30 days. After this time, the surviving animals were sacrificed and autopsied to determine the presence of tumor cells, or of a solid growth in the peritoneal cavity. Three series of experiments were performed.

Series 1.—Extracts used in this first study were made by extraction procedure 1. An effort was made to determine whether a particular cold-alcohol-precipitated fraction contained all the carcinostatic activity. We studied fractions obtained by the addition of various amounts of alcohol in the cold. A wide range of these fractions was examined, including those obtained with alcoholic concentrations from 0 to 80 percent. These fractions were then tested against the mouse tumor.

Series 2.—The second series of experiments was designed to test extraction procedures 2, 3, 4, and 5.

Series 3.—In this series we tested purified fractions of extraction procedures 4 and 5 as noted above.

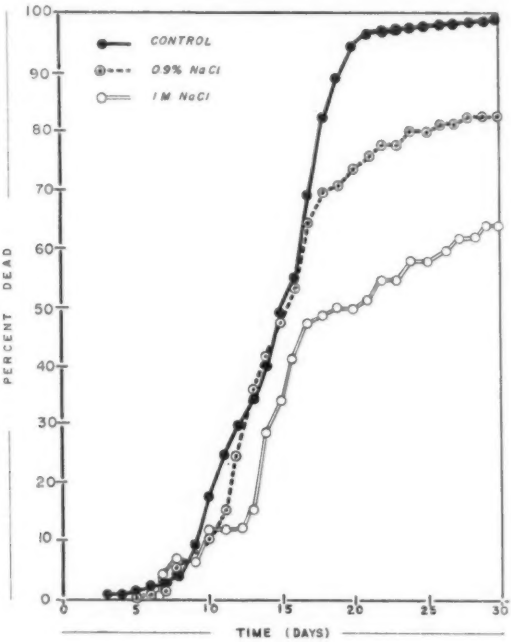
Results

A. The Control Animals

In 12 separate experiments in which mice were inoculated with the test dosage of tumor (1.5×10^6 T cells in 0.2 ml.), an average of 99 percent of the control animals had died at the end of 30 days. A total of 285 animals were used in the 12 control groups. The cumulative mean percent of dead mice with respect to time in days is shown in text-figures 1, 2, and 3. These values were derived from the fact that the percent of animals dead on the same day in 12 separate control groups is binomially distributed. The increment percent of control animals that died on each day of the experiment is shown in text-figures 4, 5, 6, and 7. The mean survival time of a control group is 14.9 days ± 0.8 days.[4]

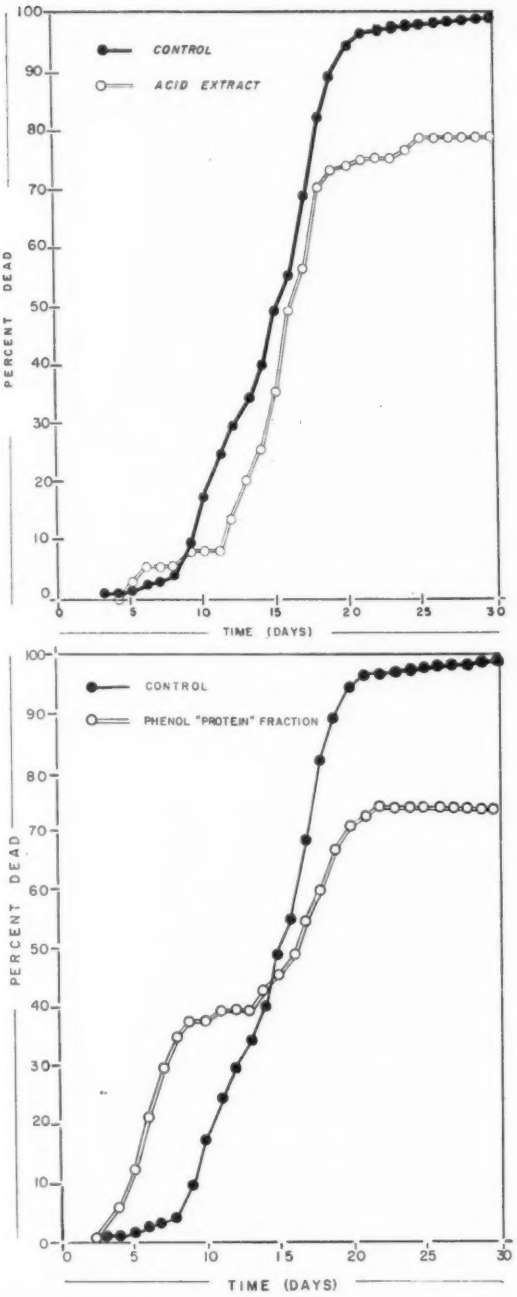
B. Series 1

The results of 4 experiments designed to indicate in which particular cold-alcohol-precipitated fraction of the aqueous extract the carcinostatic effect was located are summarized in text-figure 8. A total of 298 animals was used in the compilation of text-figure 8. An "active" fraction would have one or both of the following effects: the mean survival time of the treated animals is significantly greater at .01 level of probability than that of the control, and/or at the 30th day, when the experiment is terminated,

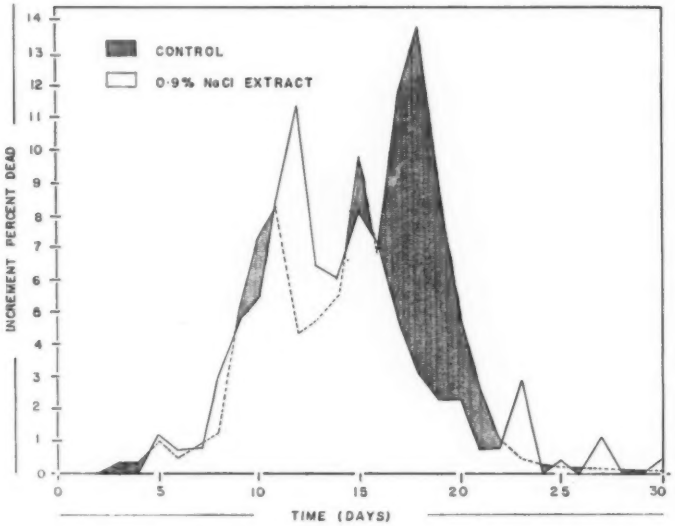


TEXT-FIGURES 1, 2, and 3.—The cumulative mean percent with respect to time in days.

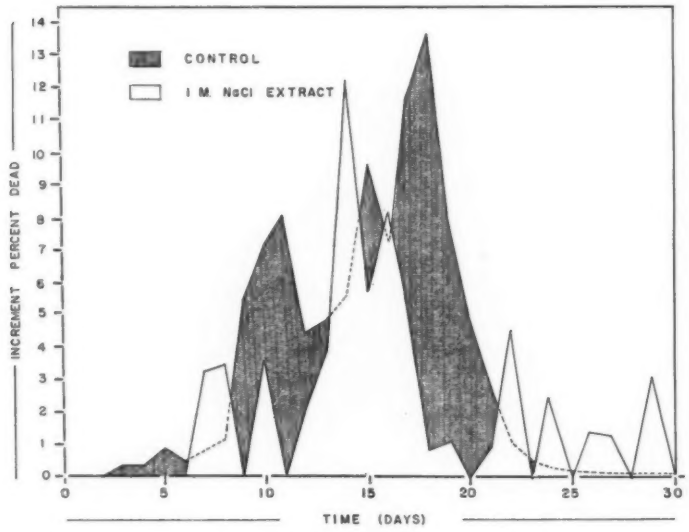
[4] ± Indicates standard error of the mean, here, and elsewhere in the text.



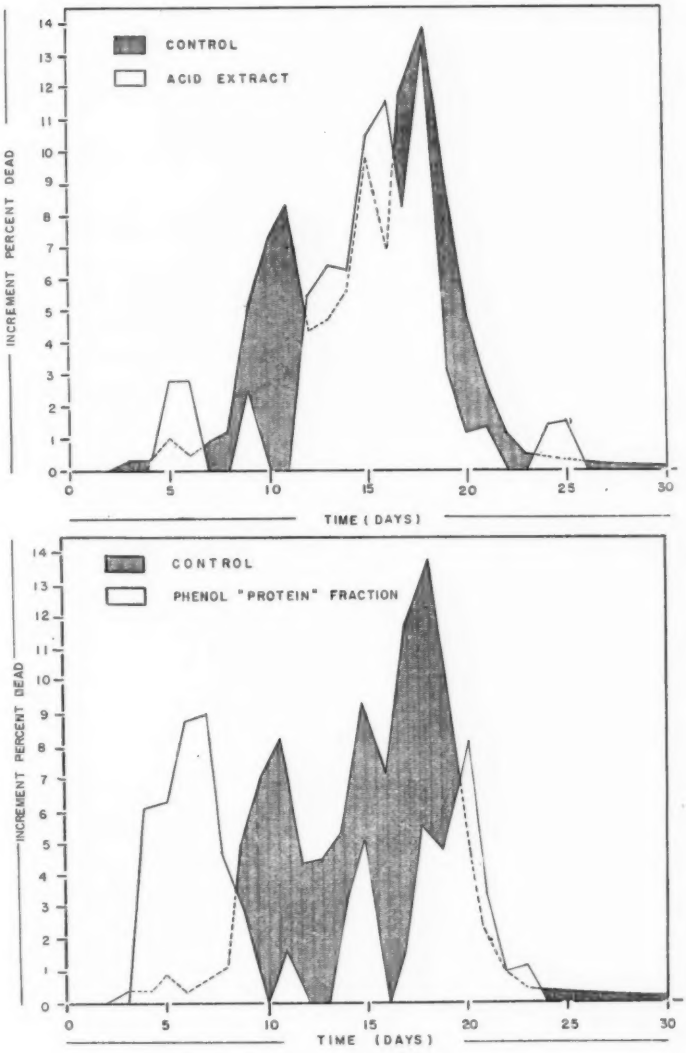
TEXT-FIGURES 2 and 3.



TEXT-FIGURES 4, 5, 6, and 7.—The *shaded* area connotes the difference between the control increment percent dead and the experimental increment percent dead when the former is greater than the latter. When the experimental increment percent dead is greater than the control increment percent dead, the control is represented by a dotted line.



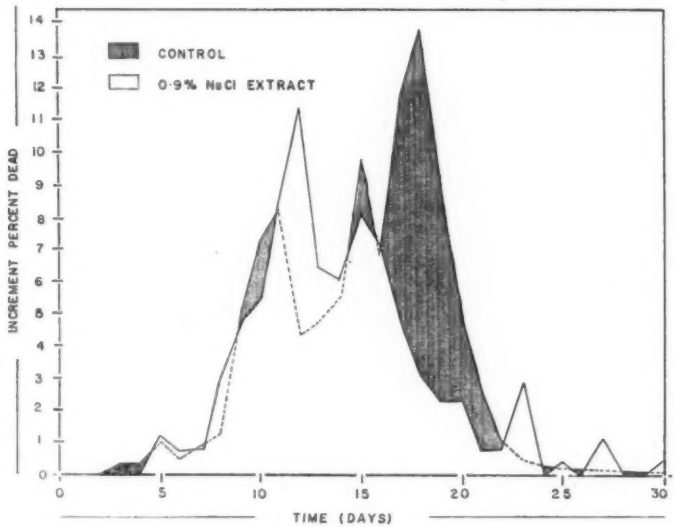
TEXT-FIGURE 5.



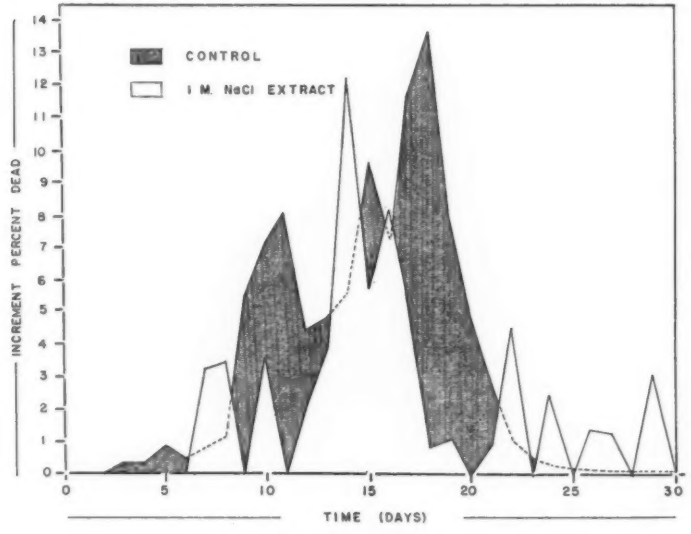
TEXT-FIGURES 6 and 7.

the percent survival of the treated group is significantly greater than the percent survival of the control on that day.

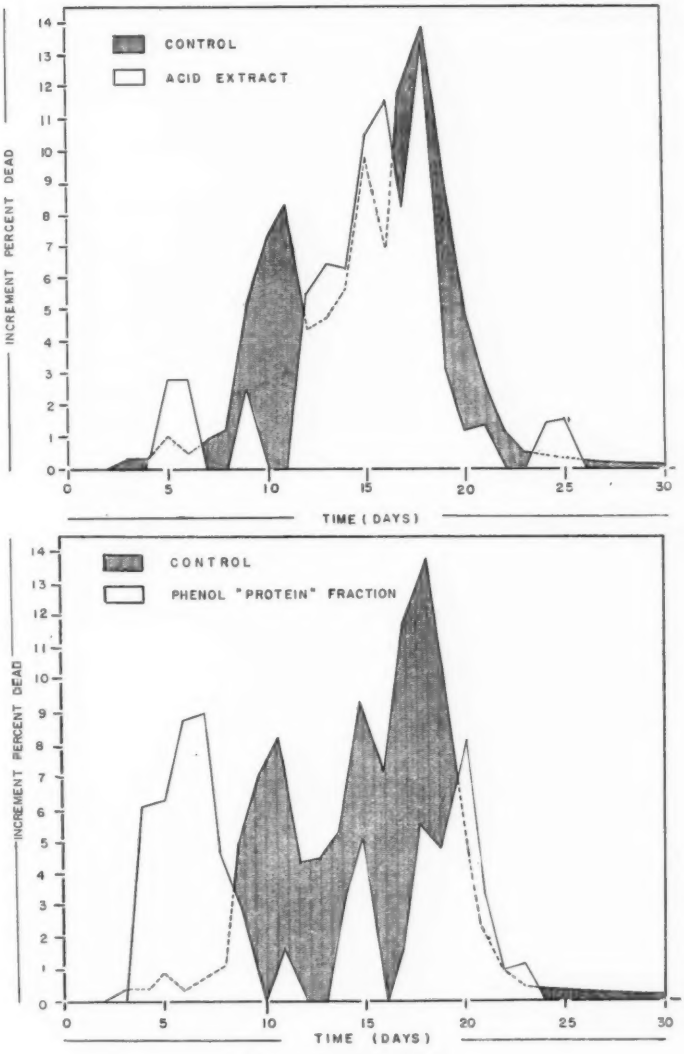
Text-figure 8 shows that the activity in the aqueous extract is located in those fractions that were precipitated with 45 to 80 percent alcohol. The mean survival time of the treated animals did not differ from the mean survival time of the control animals. On the 30th day, however, the percent survival of the treated animals is significantly greater than that of the control. On the basis of the total number of animals treated



TEXT-FIGURES 4, 5, 6, and 7.—The *shaded* area connotes the difference between the control increment percent dead and the experimental increment percent dead when the former is greater than the latter. When the experimental increment percent dead is greater than the control increment percent dead, the control is represented by a dotted line.



TEXT-FIGURE 5.



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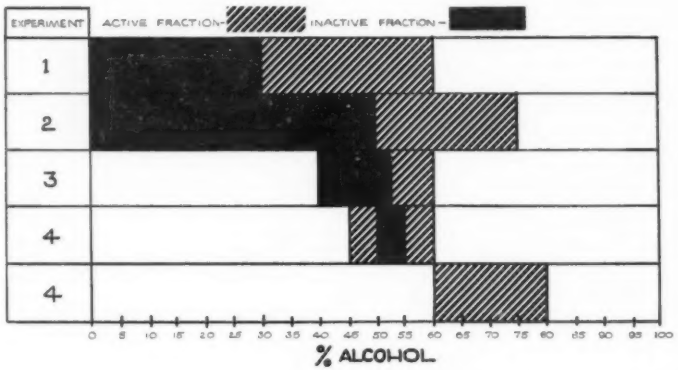

TEXT-FIGURE 8.—Summary of 4 experiments that indicate in which particular cold-alcohol-precipitated fraction of the aqueous extract the carcinostatic effect was located.

with active fractions (134), 17.4 percent survived 30 days. No tumor could be found in 15.2 percent of the animals, and the remaining 2.2 percent had solid growths in the peritoneal cavity. The difference between the experimental percent dead and the control percent dead is significant in terms of the .01 level of probability. In this case, as in subsequent results, the significance of differences in percent dead on the 30th day was derived from the confidence-coefficient tables of Clopper and Pearson (*6*).

C. Series 2

Table 2 presents the results of experiments in which a variety of extraction procedures was used. Activity was found in all alcohol-precipitated fractions of the 1 *M* NaCl extract and in 1 fraction of the acid extract. The crude basic extract and the crude phenol extract showed no activity. On the basis of the 60 animals used for experimentation with active fractions of extract #2, 35.5 percent survived 30 days, of which 30.5 percent had no apparent tumor and the remaining 5 percent had an ascites growth, the lethality of which was delayed. Of the 36 animals inoculated with active fractions of extract #3 (the acid extract), 21.6 percent survived 30 days. When the surviving animals were autopsied, no tumors were found. The mean survival of the animals in groups treated with active fractions of extracts #2 and #3 did not differ from that of the control. On the 30th day the percent surviving in these groups was significantly greater than the control. Text-figures 1 and 2 show the relationship of the percent dead *versus* the time (days) for the animals treated with the various types of extracts. Text-figures 4, 5, and 6, and table 3, show the increment percent dead by days for animals treated with the various types of extracts. The percent dead in both groups of animals treated with extracts #2 and #3 are significantly different from the control percent dead at the .01 level of probability on the 30th day.

TABLE 2.—*Results of experiments with a variety of extraction procedures*

Procedure and extract No.	Extraction medium	Alcohol used to precipitate (*percent*)	Average percent mice surviving after day: 10	15	20	30	Total No. of animals
	Control	—	82. 9	50. 1	4. 5	0. 1	265
1	0.9% NaCl	30–80	84. 2	43. 3	23. 7	17. 4	134
2	1 *M* NaCl	0–80	89. 5	65. 4	49. 5	35. 5	60
3	"Acid"	45–60	91. 9	63. 3	25. 8	21. 6	36
4	"Basic"	0–70	83. 0	50. 0	0	0	12
5	"Phenolic"	Crude	85. 0	55. 0	25. 0	0	20

D. Series 3

Text-figures 3 and 7 and table 3 give the results of series 3. In each case, the crude preparations were found to be inactive. When these preparations were purified, as described in section B of "Materials and Methods," the "protein" fraction of extract #5 was found to be active. A total of 44 animals was treated with the active fraction of extract #5 and results with the "protein" fraction of this extract showed that 25.7 percent of the treated mice survived 30 days. In all these mice no apparent tumor could be found. The percent dead, however, is again significantly different from the control percent dead at the .01 level of probability.

TABLE 3.—*Results of experiments in series 3*

Procedure and extract No.	Fraction	Average percent mice surviving after day: 10	15	20	30
Control		82. 9	50. 1	4. 5	0. 1
4	Crude	83. 0	50. 0	0	0
	Protein	83. 0	42. 0	0	0
5	Crude	85. 0	55. 0	25. 0	0
	Protein	80. 0	52. 1	31. 8	25. 7

Discussion

The effective fractions of the aqueous extract were those obtained by precipitation in higher percentages of alcohol. Extraction by acid and by 1 *M* NaCl gave fractions that likewise exhibited a significant effect on the lethality of the tumor. Purification of an extract that was inactive in the crude state in one case, *i.e.*, phenol partition, gave an effective fraction. Far from being destroyed by the removal of loosely bound lipides and nucleic acids, the activity actually appeared only after their removal.

Text-figures 4, 5, 6, and 7 show the relationship of the increment percent dead, of tumorous control animals, against time, compared to similar data for animals treated with various extracts. In the control animals the tumor has a maximum lethality centered about the 18th day, with a slight peak in "killing effectiveness" on the 10th day. Table 4 summarizes this data for the control animals and for those animals treated with active extracts, as well as for those treated with inactive extracts. The numbers in the table represent the increment percent dead on that day (within the interval of days cited at the top of the table) on which the maximum number of animals died. This figure is therefore the "maximum increment percent dead" within the respective interval of days. Hence between the 7th and 13th days, of the control animals, the maximum increment percent dead on any single day was 8.4 percent. The 4 intervals of time used in the table were selected arbitrarily. The "inactive" fractions of the 0.9 percent NaCl extract were those precipitated by the addition of ethyl alcohol to a final concentration of 40 percent. These fractions exhibited no effect when tested against the lethality of the tumor in the mice.

TABLE 4.—*Summary data for control mice and mice treated with extracts*

Extract group	The maximum increment percent dead between days: 0–6	7–13	14–20	21–27
Control	1.0	8.4	13.8	2.6
Inactive 0.9% NaCl	2.8	18.3	12.3	0.2
Active 0.9% NaCl	1.1	11.4	8.3	2.9
1 *M* NaCl	0	3.9	12.3	4.5
Acid	2.8	6.4	13.5	1.6
Crude phenol	10.0	5.0	15.0	15.0
Phenol "protein"	8.8	9.1	8.1	3.5

Qualitatively the extracts alter the pattern of the lethality of the tumor. Both the active fractions of the 0.9 percent NaCl extract and the "protein" fraction of the phenolic extract enhance the early lethality of the tumor as compared to the lethality of the tumor in the control animals During the interval between days 14 to 20, however, when the control tumor has reached its peak in lethality, animals treated with these extracts exhibit a reduced lethality. Animals treated with the 1 *M* NaCl extract show a reduced lethality in the first 3 intervals of time followed by a subsequent rise in lethality between the 21st and 27th days. The animals treated with the acid extract, on the other hand, exhibit a relatively increased lethality in the first interval of time and a decreased lethality, compared to the control, in the successive intervals of time (*see* table 3).

The mean survival time of the control animals was 14.9 days $\pm$ 0.8 days. Allowing for the difference in the number of animals in the experi-

mental group as compared to the control group, there is no significant difference between the mean survival time of the control and that of the animals treated with active fractions of 0.9 percent NaCl (13.7 days ± 1.3 days), the 1 *M* NaCl extract (15.8 days ± 2.7 days), the acid extract (15.2 days ± 2.2 days), and the mean survival time of the animals treated with the "protein" fraction of the phenolic extract, which was 12.0 days ± 2.1 days.

Toxicity controls, in which nontumorous animals were inoculated with the same dosage of extract given to tumorous animals, show no evidence of toxicity. In most of the treated animals that survived after 30 days, autopsy failed to reveal signs of tumor either in ascites form or in the form of solid growths in the peritoneum. If the extract has the effect of destroying a certain number of tumor cells within the first 5 days of tumor growth, then one might consider that the products of this destruction are relatively lethal to the animal before the 13th day of the experiment. Hence, one finds the greater early lethality exhibited by the tumor in the treated animals. Only with the 1 *M* NaCl extract is this effect not present. One might consider then, that an active extract destroys tumor cells, and the animals capable of surviving the toxicity of the products of this destruction are the survivors found at the end of 30 days. The authors have observed experimentally that the products of disrupted tumor cells, when inoculated into tumorous animals, do, in fact, enchance the early lethality of the tumor (7).

Summary

1) A carcinostatic effect of a variety of types of extracts of cow ovaries has been studied. This effect was measured in terms of the survival of mice inoculated with a Ehrlich ascites tumor and subsequently treated with extracts.

2) The control animals inoculated with 1.5×10^6 cells, in 12 separate groups, totaled 285. The mean survival time of a control group was 14.9 ± 0.8 days.

3) Tumorous animals treated with the aqueous extract showed a survival of 17.4 percent at the end of 30 days. Animals treated with the 1 *M* NaCl extract showed a survival of 35.5 percent at the end of 30 days. Animals treated with an acid extract showed a survival of 21.6 percent. All 3 of these figures are significantly different from the control.

4) An inactive crude preparation was prepared by phenol partition. This crude material was purified by the removal of loosely bound lipides and nucleic acids. After purification the preparation had a carcinostatic effect. Animals treated with the purified preparation exhibited a survival of 25.7 percent at the end of 30 days.

5) The mean survival time of a group of treated animals is not significantly different from the mean survival time of the control group.

6) The distribution of the daily lethality of the tumor was shifted in the case of the extract-treated animals. This change generally was to enhance the lethality of the tumor during the early days of tumor growth.

7) An explanation of the enhancement of the early lethality of the tumor in the treated animal was suggested in terms of the toxicity of the products of disrupted tumor cells.

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Effects of Antitumor Compounds and Radiation on the Glycolysis and Respiration of Ascites-Tumor Cells[1]

TOSHIAKI EBINA *and* MORIJI KUROSU,[2] *The Research Institute for Tuberculosis and Leprosy, Tohoku University, Sendai, Japan*

Since the first description by Loewenthal and Jahn (*1*), and the work of Lettre (*2*), Klein (*3*), and Yoshida (*4, 5*) on mouse ascites tumors, it has been possible to study the influence of drugs on the metabolism of tumors. The technique provides a readily obtainable and almost pure culture of tumor cells.

Determinations of respiration and glycolysis of mouse ascites-tumor cells have been reported by Warburg (*6–8*) and Kun *et al.* (*9*). Mihich, Clarke, and Philips (*10*) studied the effects of 6-mercaptopurine on respiration and anaerobic glycolysis in normal and tumor tissues and found that treatment for 7 days with tolerated doses caused a reduction of 50 percent or more in the respiration and anaerobic glycolysis of sarcoma 180, but that there was no impairment of the metabolic function in liver and kidney. In this study an attempt was made to investigate the inhibitory effects of 9 well-known antitumor compounds, 14 allyl derivatives of uncertain antitumor efficacy, and the effect of X irradiation on respiration and glycolysis of ascites-tumor cells.

Materials and General Methods

The ascites form of three tumors, Yoshida ascites sarcoma, Ehrlich ascites carcinoma, and rat ascites hepatoma was used. The respiration and glycolysis were determined by the standard manometric technique of Warburg.

The experimental animals were killed at the time of full multiplication of ascites-tumor cells—5 to 8 days. The ascitic fluid was withdrawn with a pipette, diluted with 0.9 percent saline, and then centrifuged at 1,000 $\times$ g for 5 to 10 minutes. After the 2nd washing, the tumor cells were again suspended in 0.9 percent saline so that 0.2 ml. of the suspension contained about 4 to 10 mg. in dry weight of tumor cells. This procedure took about 2 hours until the readings of the manometers were begun.

Respiration experiments were conducted with Krebs-Ringer-phosphate buffer, while in glycolysis experiments the medium was Krebs-Ringer-

[1] Received for publication September 10, 1957.

[2] The authors wish to express their appreciation to Dr. Kanematsu Sugiura, Sloan-Kettering Institute for Cancer Research, New York, for sending us valuable antitumor compounds used in these studies.

bicarbonate buffer. The concentration of glucose was 0.2 gm. per 100 ml.

The respiration of the cells was determined manometrically with 0.2 ml. of 20 percent KOH in the center well of each Warburg flask. Each flask contained 2.0 ml. of Krebs-Ringer-phosphate buffer and 0.2 ml. of cell suspension. The gas phase was pure oxygen. After 10 minutes of temperature equilibration, readings were made every 15 minutes for at least 2 hours.

Anaerobic glycolysis was determined manometrically in Krebs-Ringer-bicarbonate solution with 95 percent N_2 and 5 percent CO_2 as the gas phase. Each flask contained 2.0 ml. of Krebs-Ringer-bicarbonate solution and 0.2 ml. of cell suspension.

Aerobic glycolysis was calculated from the readings of 2 flasks which contained 2 and 8 ml. of Krebs-Ringer bicarbonate, respectively.

All measurements of respiration and glycolysis were conducted at 37.5° C., and the shaking frequency was 120 per minute. Room temperature was about 15° C.

The procedures were all done asceptically and the contents of the flasks were tested for sterility at the end of each experiment. When many colonies were found on agar slants, the results were not included. In each experiment, after treatment with the chemicals or after exposure to X irradiation, some of the cell material was smeared and stained by Giemsa's method to observe any morphological change. Part of the cell suspensions was transplanted to animals for the vitality test and 0.2 ml. was dried on a watch glass at 70° C. for 1 hour for dry-weight determination.

Experimental Results

Experiment 1

The mean quotients of respiration and glycolysis of the 3 types of ascites-tumor cells taken from 12 different samples and examined in the absence of antitumor compounds or X irradiation are shown in table 1. The tissue metabolism of the 3 tumors showed almost the same value but the $Q_M^{N_2}$ of the Yoshida ascites sarcoma cells was slightly lower and the QO_2 of the rat ascites hepatoma cells was somewhat higher. High aerobic and anaerobic glycolysis was always observed in all experiments.

Experiment 2

The effect of 9 drugs on tissue metabolism *in vitro* was studied. The 9 antitumor agents used were as follows: Methyl-bis(β-chloroethyl)amine *N*-oxide hydrochloride,[3] (Nitromin), 3-bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine (SK 1424), Sarkomycin, 8-azaguanine, aminopterin, amethopterin, 1,9-di(methanesulfonoxy) nonane,[4] 6-mercaptopurine (6-MP), and triethylenethiophosphoramide (thio-TEPA). The compounds were dissolved in Krebs-Ringer-phosphate

Chemical Abstracts nomenclature:

[3] 2,2'-Dichloro-*N*-methyldiethylamino *N*-oxide hydrochloride.

[4] 1,9-Bis(methylsulfonyloxy)nonane.

TABLE 1.—*Respiratory and glycolytic quotients of intact ascites-tumor cells**

	Yoshida ascites sarcoma			Ehrlich ascites carcinoma			Rat ascites hepatoma		
	QO_2	$Q_M^{O_2}$	$Q_M^{N_2}$	QO_2	$Q_M^{O_2}$	$Q_M^{N_2}$	QO_2	$Q_M^{O_2}$	$Q_M^{N_2}$
	5.5	—	35.6	8.2	—	40.5	8.7	—	25.6
	6.2	—	29.5	6.0	20.3	36.7	9.0	25.4	43.9
	5.9	—	43.0	5.5	10.7	38.0	7.7	18.7	39.6
	5.0	—	32.1	7.8	28.5	42.8	5.8	16.2	32.4
	7.3	—	28.8	6.6	17.9	28.1	5.6	22.4	61.3
	8.6	—	35.3	3.0	16.5	35.2	5.9	20.4	51.9
	6.1	19.1	24.8	5.0	25.2	35.9	5.6	13.3	27.3
	6.6	19.7	30.0	6.6	36.4	39.0	4.7	33.0	31.5
	7.6	20.8	26.2	7.3	—	36.6	14.6	—	25.3
	3.6	14.9	21.0	5.9	—	36.0	17.6	—	25.6
	4.5	23.0	34.9	7.0	—	36.6	13.0	—	43.7
	6.7	—	34.6	12.5	—	38.3	4.7	—	36.5
Mean	6.1	19.5	31.3	6.8	23.4	37.0	8.6	21.0	37.0
M.O.Q.	5.8			6.1			5.6		

*QO_2=μl. of O_2 consumed per mg. in dry weight per hour.

$Q_M^{O_2}$=μl. of lactic acid produced aerobically per mg. in dry weight per hour.

$Q_M^{N_2}$=μl. of lactic acid produced anaerobically per mg. in dry weight per hour.

M.O.Q.=Meyerhof oxidative quotients, $3 \times \frac{Q_M^{N_2} - Q_M^{O_2}}{QO_2}$.

solution to make a concentration of 10γ per ml. The *p*H was adjusted to 7.4, and 2 ml. of the solution was added to each Warburg flask which contained 0.2 ml. of the cell suspension. Technical details were the same as described, except for addition of drugs or X irradiation. In this experiment each test had its own control. The effects of several antitumor compounds on respiration and glycolysis, at a concentration of 10γ per ml., showed no inhibition. Perhaps such a low concentration cannot have an effect upon tissue metabolism *in vitro* within a period of 2 hours.

Next, the effects of drugs at higher concentrations of 100 and 1000γ per ml. were compared. Sarkomycin inhibited the respiration and anaerobic glycolysis of Yoshida ascites sarcoma cells, 38.8 and 50.3 percent, respectively, at 1000γ per ml. The corresponding figures at 100γ per ml. were 21.5 and 12.2 percent.

Nitromin restrained the respiration and anaerobic glycolysis of Ehrlich ascites carcinoma cells at 1000γ per ml., 79.1 and 79.8 percent; at 100γ per ml., 46.6 and 51.4 percent, respectively. Amethopterin inhibited the respiration and anaerobic glycolysis of Ehrlich ascites carcinoma cells at 1000γ per ml., 51.9 and 16.5 percent; at 100γ per ml., 8.9 and 3.0 percent, respectively.

The same phenomena could be seen in all tests using the 3 tumors and 9 drugs. The effects of the 9 antitumor compounds are shown in table 2. We describe inhibitory effects of drugs upon tissue metabolism

TABLE 2.—*Inhibitory percentage of antitumor compounds on the respiratory and glycolytic quotients of ascites-tumor cells*

Compounds	Dose (γ/*ml.*)	Yoshida ascites sarcoma			Ehrlich ascites carcinoma			Rat ascites hepatoma		
		QO_2 (*percent*)	$Q_M^{O_2}$ (*percent*)	$Q_M^{N_2}$ (*percent*)	QO_2 (*percent*)	$Q_M^{O_2}$ (*percent*)	$Q_M^{N_2}$ (*percent*)	QO_2 (*percent*)	$Q_M^{O_2}$ (*percent*)	$Q_M^{N_2}$ (*percent*)
Nitromin	1000	56. 4	44. 1	45. 7	79. 1	75. 8	79. 8	72. 8	45. 8	52. 1
	100	36. 3	24. 6	16. 2	46. 6	56. 7	51. 4	49. 2	24. 5	48. 0
SK-1424	1000	46. 3	32. 7	42. 1	38. 4	59. 0	53. 8	31. 6	41. 9	35. 5
	100				7. 8	14. 5	7. 1			
Sarkomycin	1000	38. 8	49. 6	50. 3	65. 3	61. 4	79. 9	67. 6	63. 8	68. 4
	100	21. 5	21. 5	12. 2	12. 8	15. 8	20. 4	7. 9	25. 8	25. 1
8-Azaguanine	1000	6. 4	9. 2	6. 9	3. 7	10. 7	15. 1	1. 0	31. 3	39. 2
	100				1. 0	4. 0	2. 3			
Aminopterin	1000				58. 2		42. 0			
	100				18. 8	3. 3	8. 6			
Amethopterin	1000				51. 9		16. 5			
	100				8. 9		3. 0			
1,9-Di(methanesulfonoxy)nonane	1000	55. 8	−65. 3	−1. 0	92. 5	−83. 5	6. 0	83. 4	−80. 0	−2. 0
6-Mercaptopurine	1000	11. 4	−17. 5	−6. 5	0. 5	−1. 8	7. 3	−1. 0	1. 2	13. 2
Triethylenethiophosphoramide	1000	12. 7	4. 3	6. 2				11. 9	8. 3	5. 0

as "strong" when its percentage compared with its own control was over 70 percent, as "moderate" when its percentage was between 70 and 40 percent, and as "weak" when it was between 40 and 20 percent.

As table 2 shows, Nitromin inhibited the respiration (QO_2) and the anaerobic glycolysis ($Q_M^{N_2}$) of Yoshida ascites sarcoma cells moderately; of Ehrlich ascites carcinoma cells strongly; and of rat ascites hepatoma cells strongly or moderately. SK 1424 also inhibited the QO_2 and $Q_M^{N_2}$ of Yoshida ascites sarcoma and Ehrlich ascites carcinoma cells moderately and those of rat ascites hepatoma cells weakly. Sarkomycin depressed the metabolism of these 3 tumors moderately or strongly. 8-Azaguanine did not have any significant influence except for weak inhibition of QO_2 and $Q_M^{N_2}$ of rat ascites hepatoma cells. Aminopterin moderately inhibited both QO_2 and $Q_M^{N_2}$ of Ehrlich ascites carcinoma cells but had no effect upon $Q_M^{O_2}$. 1,9-Di(methanesulfonoxy)nonane inhibited QO_2 of ascites-tumor cells of the three types strongly, increased $Q_M^{O_2}$ up to 60 or 80 percent, but had no influence on $Q_M^{N_2}$. 6-Mercaptopurine and thio-TEPA seemed to have no significant influence.

Fourteen allyl derivatives, synthesized by Dr. Hideo Endo, of this Institute, were also tested for their effect on the respiration and glycolysis of Yoshida ascites sarcoma cells at the concentration of 1000γ per cc. The results are illustrated in table 3. β-Allylaminonaphthalene and α-allylaminonaphthalene inhibited the QO_2 and $Q_M^{N_2}$ to the extent of 60 to 70 percent. Allyliminothiadiazoline and 6-allylaminoquinoline inhibited the QO_2 and $Q_M^{N_2}$ moderately. Allylaminobenzene, *N*-allylthiourea, 4-allylthiosemicarbazide and allylimino-C-methylthiadiazoline inhibited the QO_2 moderately but did not influence the $Q_M^{N_2}$. 4-Allylaminopyridine inhibited the QO_2 markedly and promoted $Q_M^{N_2}$ moderately. *p*-Bis(allylamino)benzene, 2-allylaminopyridine, *p*-allylaminobenzylcyanide, acetylallylamine, and 4-allyl-1-formylthiosemicarbazide had no perceptible effect on either QO_2 or $Q_M^{N_2}$.

For purposes of comparison, the inhibitory effects of these compounds on Ehrlich solid carcinoma, as observed by Dr. Endo (*11*), are included in table 3.

To observe the effects of roentgen radiation, the Yoshida ascites sarcoma cells in each flask were X-irradiated and the tissue metabolism was measured. Irradiating conditions were as follows: Tube potential 160 kvp 3 ma.; filter 1.0 mm. Al + 0.5 mm. Cu; half value layer 1.02 mm. Cu; distance 30 cm.; dose rate 20 r per minute. The QO_2 and $Q_M^{N_2}$ of Yoshida ascites sarcoma cells were not considerably depressed after X irradiation with 1000 r of X rays given once (table 4).

Experiment 3

The influence of compounds on the tumor metabolisms *in vivo* was determined. The compounds were injected into the abdominal cavity of rats having Yoshida ascites sarcoma. Twenty-four hours after injection, the rats were sacrificed and the glycolysis and respiration of the ascites-tumor cells were measured with no further addition of drugs to the flask.

TABLE 3.—*Effects of allyl compounds on the metabolic quotients of Yoshida ascites sarcoma cells (inhibitory percentage at 1 hour.—compounds were applied* in vitro)

Compounds (*1000 γ per ml.*)	Inhibitory percentage		Tumor inhibitory percentage*
	QO_2	$Q_M^{N_2}$	
Allylaminobenzene	42. 1	8. 8	50–70 (*200mg./kg.*)
p-Bis(allylamino)benzene	5. 8	−10. 4	25–50 (*50mg./kg.*)
β-Allylaminonaphthalene	70. 5	70. 0	50–70 (*400mg./kg.*)
α-Allylaminonaphthalene	59. 9	71. 5	25–50 (*200mg./kg.*)
4-Allylaminopyridine	57. 6	−20. 0	25–50 (*25mg./kg.*)
2-Allylaminopyridine	10. 4	−5. 0	>25 (*100mg./kg.*)
Allyliminothiadiazoline	48. 1	32. 2	90–100 (*100mg./kg.*)
6-Allylaminoquinoline	64. 6	49. 5	65–75 (*100mg./kg.*)
p-Allylaminobenzylcyanide	−5. 8	−3. 0	25–50 (*50mg./kg.*)
N-Allylthiourea	28. 0	−6. 0	25–50 (*200mg./kg.*)
Acetylallylamine	12. 8	3. 0	25–50 (*200mg./kg.*)
4-Allylthiosemicarbazide	44. 7	4. 6	25–50 (*10mg./kg.*)
4-Allyl-1-formylthiosemicarbazide	−4. 0	6. 3	25–50 (*200mg./kg.*)
Allylimino-C-methylthiadiazoline	32. 8	2. 6	25–50 (*200mg./kg.*)

*Tumor inhibitory effect is quoted from the results of experimental chemotherapy by Endo on Ehrlich solid carcinoma. Each agent was injected intraperitoneally at the doses of tolerable maximum for 2 weeks. The numbers in parentheses are doses of drugs.

The effects of Nitromin, Sarkomycin, 8-azaguanine, 1,9-di(methanesulfonoxy)nonane, 6-mercaptopurine, and thio-TEPA on the tissue metabolism of Yoshida ascites sarcoma cells 24 hours after a single intraperitoneal injection are shown in table 5. Because all tumor cells taken from once-treated rats were under the influence of that drug, each test could not have its own control. Therefore, mean data for several determinations were compared with the author's mean tissue metabolism of Yoshida ascites sarcoma (*see* table 1) to find the inhibitory percentage. Nitromin, Sarkomycin, 1,9-di(methanesulfonoxy)nonane and thio-TEPA had a moderately promotive effect on the tissue metabolism of Yoshida

TABLE 4.—*Inhibitory percentage of X rays (γ rays) on the respiratory and glycolytic quotients of ascites-tumor cells*

Cells / Q. / X-ray doses	Yoshida ascites sarcoma	
	QO_2	$Q_M^{N_2}$
500γ	2. 2 3. 8 4. 0 5. 4	17. 0 15. 0 8. 0 12. 0
Mean	3. 9 Percent	13. 0 Percent
1000γ	7. 0 8. 4 14. 0 16. 0	28. 2 27. 0 19. 0 17. 0
Mean	11. 3 Percent	22. 4 Percent

ascites sarcoma cells. On the other hand, 8-azaguanine and 6-mercaptopurine caused some inhibition.

To determine the influence of β rays, 700 μc of Au^{198}, in 20 percent glucose solution, was injected intraperitoneally into rats 3 days after transplantation of Yoshida ascites sarcoma. Twenty-four hours after injection, the glycolysis and respiration of ascites-tumor cells were measured. When the mean data for several determinations were compared with the author's mean tissue metabolism of Yoshida ascites sarcoma, it was found that Au^{198} had a markedly promotive effect on the tissue metabolism, even on the 5th day after the intraperitoneal injection (table 6).

Experiment 4

The inhibitory effect of these compounds on the growth of Ehrlich solid carcinoma was also examined. Chemotherapy was begun on the day after hypodermic inoculation, and drugs were given intraperitoneally every day for 2 weeks when the animals were sacrificed and tumors weighed.

Nitromin showed marked inhibitory effect on the growth of Ehrlich solid carcinoma; Sarkomycin, 1,9-di(methanesulfonoxy)nonane, and 9-mercaptopurine were also moderately effective. Amethopterin, however, showed no effect (table 7).

After the termination of the measurements in each experiment, the tumor cells in flasks were examined for viability and in all tests no loss of vitality could be found. Morphologically, also, no significant changes were observed in the smear of tumor cells except for 6-mercaptopurine. Yoshida ascites sarcoma cells, at 24 hours after an intraperitoneal injection of this drug, showed more cytoplasmic and nuclear vacuolization than normal tumor cells.

TABLE 5.—*Effects of antitumor compounds on tissue metabolism of Yoshida ascites sarcoma cells 24 hours after a single intraperitoneal injection*

Compounds	Nitromin		Sarkomycin		8-Azaguanine		1,9-Di(methane-sulfonoxy)nonane		6-Mercaptopurine		Thio-TEPA	
Doses	(*30 mg./kg.*)		(*300 mg./kg.*)		(*500 mg./kg.*)		(*100 mg./kg.*)		(*100 mg./kg.*)		(*2.5 mg./kg.*)	
Quotients	QO_2	$Q_M^{N_2}$	QO_2	$Q_M^{N_2}$	QO_2	$Q_M^{N_2}$	QO_2	$Q_M^{N_2}$	QO_2	$Q_M^{N_2}$	QO_2	$Q_M^{N_2}$
Expt. No. 1	8. 7	39. 5	7. 5	34. 0	4. 2	27. 5	8. 2	43. 8	2. 9	19. 2	9. 3	39. 0
2	8. 4	37. 8	8. 8	34. 5	4. 8	27. 8	8. 8	44. 5	6. 4	27. 5	8. 6	47. 7
3	7. 3	36. 4	9. 0	36. 8	5. 3	28. 5	8. 9	47. 3	7. 6	28. 7	9. 6	44. 0
4	6. 8	36. 1	9. 2	36. 6	4. 7	30. 9	8. 9	48. 7	3. 9	24. 4		
5	6. 9	35. 6	9. 4	37. 0	5. 4	32. 0	9. 3	50. 4	6. 2	24. 0		
					6. 0	34. 4						
Mean	7. 9	37. 1	8. 8	35. 8	5. 7	30. 2	8. 8	46. 9	5. 4	24. 8	9. 2	43. 5
Inhibitory percentage*	−30. 0	−19. 0	−44. 0	−14. 0	6. 5	3. 5	−44. 0	−48. 0	11. 3	20. 7	−51. 0	−39. 0

*Minus inhibitory percentage means percentage of promoting effect.

TABLE 6.—*Effect of Au^{198} (β rays) on tissue metabolism on the 1st and 5th day after single intraperitoneal dose of 700 μc*

Cells / Q. / Day after injection	Yoshida ascites sarcoma	
	QO_2	$Q_M^{N_2}$
1st day	11.6 11.8 12.6 12.7 12.8	51.0 51.2 51.4 51.8 52.5
Mean	12.3	51.6
Inhibitory percentage	−102.0	−65.0
5th day	9.2 10.1 11.3	44.3 44.5 45.2
Mean	10.2	44.8
Inhibitory percentage	−68.0	−43.0

Discussion

Warburg (*6–8*) suggested that the best measurement of the respiration and glycolysis of free ascites-tumor cells was a method using ascitic serum as medium. According to his report, the respiratory quotient and the aerobic glycolytic and anaerobic glycolytic quotients of mouse ascites-tumor cells were 7, 30, and 70, respectively.

In our experiments the medium employed was Krebs-Ringer-phosphate buffer for respiration and Krebs-Ringer-bicarbonate buffer for glycolysis determinations. We found the glycolytic quotients of 3 ascites-tumor cells were lower than those of Warburg's experiments. Kun *et al.* (*9*) used almost the same method, and the quotients of the tissue metabolism of Ehrlich ascites carcinoma cells were described as QO_2 8.1 and $Q_M^{N_2}$ 48.2.

These quotients were nearer the results obtained with slices of Jensen sarcoma or Flexner-Jobling carcinoma reported by Warburg (*12*), Murphy (*13*), Crabtree (*14*), and Ebina (*15*).

The tumor-inhibitory effect of Nitromin (*16*, *19*) and Sarkomycin (*20*, *21*) has been confirmed in our experiment using Ehrlich ascites carcinoma. These compounds inhibited the respiration and glycolysis of ascites-tumor cells of the 3 types of tumors either strongly or moderately when applied *in vitro*. However, glycolysis and respiration of Yoshida ascites sarcoma cells withdrawn from the abdominal cavity 24 hours after a single intraperitoneal injection of these compounds were not inhibited but rather accelerated.

TABLE 7.—*Effects of antitumor compounds on Ehrlich solid carcinoma*

Compounds	Intraperitoneal administration (*mg./kg./day*)	Number of mice	Number of mice dying	Average tumor	Inhibitory percentage
Nitromin	50 for 8 days	10	2	0. 71	74
Sarkomycin	500 for 2 days 100 for 8 days	10	7	1. 76	37
8-Azaguanine	500 for 3 days 100 for 7 days	10	2	2. 38	14
SK 1424	5 for 10 days	10	10	—	—
Amethopterin	0.5 for 10 days	10	2	3. 46	0
1,9-Bis(methanesulfonoxy)nonane	50 for 10 days	10	3	1. 86	33
6-Mercaptopurine	30 for 10 days	10	2	1. 8	35
Control		10	0	2. 78	—

8-Azaguanine (*22*, *23*) had no influence on the tissue metabolism of tumor cells other than a weak inhibition of glycolysis of rat ascites hepatoma cells *in vitro*. Finkelstein *et al.* (*24*) reported that 8-azaguanine had no effect on the respiration of slices of sarcoma 37 *in vitro* but that after intraperitoneal injection it inhibited the respiration of tumor slices by 42 to 92 percent. In our experiments, a single intraperitoneal injection of a 500 mg. per kg. dose of 8-azaguanine had no effect on QO_2 and $Q_M^{N_2}$ after 24 hours.

The strong tumor-inhibitory effect of aminopterin and amethopterin (*25*, *26*) had been reported as the action of an antimetabolite against folic acid. In our experiments, aminopterin moderately inhibited the QO_2 and $Q_M^{N_2}$ of Ehrlich ascites carcinoma cells. Amethopterin inhibited QO_2 moderately and had no effect on $Q_M^{N_2}$ *in vitro*.

1,9-Di(methanesulfonoxy)nonane (*27*, *28*) had a different mode of action. It strongly depressed the respiration of ascites-tumor cells and promoted aerobic glycolysis 60 or 80 percent but had no influence on anaerobic glycolysis *in vitro*. After intraperitoneal injection, this compound accelerated the QO_2 and $Q_M^{N_2}$ of Yoshida ascites sarcoma cells considerably. The tumor-inhibitory effect of this drug was also observed.

6-Mercaptopurine (*28–32*) had no perceptible effect on the tissue metabolism of ascites-tumor cells *in vitro*. Clarke *et al.* (*31*) reported a considerable inhibitory effect of 6-mercaptopurine on the growth of sarcoma 180 in mice. In our experiments, the growth of Ehrlich solid tumor was inhibited slightly by 6-mercaptopurine. According to Mihich *et al.* (*10*), tumor slices obtained from animals treated with 6-mercaptopurine showed that the drug depressed QO_2 and $Q_M^{N_2}$ values 50 percent. In our *in vitro* experiments, 6-mercaptopurine showed no remarkable effect on cell metabolism 24 hours after an intraperitoneal injection of 100 mg. per kg.

Thio-TEPA (*33*) had little effect on the tissue metabolism of Yoshida ascites sarcoma and rat ascites hepatoma cells *in vitro*. After an intraperitoneal injection, it promoted the QO_2 and $Q_M^{N_2}$ of Yoshida sarcoma cells considerably. We see here distinct differences between the second and third experiments in the inhibitory effects of drugs on the metabolism of cancer cells. In the second experiment most drugs showed inhibitory effects on respiration and glycolysis, but in the third experiment a promotive effect was more often observed.

What were the causes of these differences? Differences in the concentration of drugs and the time factor may be considered as causative factors. Further experiments will be required to solve this problem.

Tanaka (*34*) reported that a 600 r dose of X irradiation had no effect on the oxygen consumption of Yoshida ascites sarcoma cells. In our study a 1000 r dose of X rays had no effect on the QO_2 and $Q_M^{N_2}$ of Yoshida ascites sarcoma cells. However, the QO_2 and $Q_M^{N_2}$ of Yoshida ascites sarcoma cells were greatly accelerated at 24 hours and even 5 days after an intraperitoneal injection of 700μc of Au^{198}.

It is very difficult to ascertain the relationship between inhibitory action

upon tissue metabolism of Ehrlich ascites-tumor cells and growth-inhibitory effect upon Ehrlich solid tumor. Our experiments were not enough to solve this difficult question, but they demonstrated that Nitromin and Sarkomycin, which strongly or moderately inhibited the QO_2 and $Q_M^{N_2}$ of Ehrlich ascites-tumor cells *in vitro*, and 1,9-di(methanesulfonoxy)nonane, which inhibited QO_2 strongly, also had marked inhibitory effects on the growth of Ehrlich solid tumor.

The antitumor effect of 14 water-soluble, allyl-substituted compounds were examined by Dr. Endo (*11*), synthesizer of these drugs, who used subcutaneous solid tumors of the Ehrlich ascites carcinoma. Saline solutions of drugs were injected every day intraperitoneally for 2 weeks, and tumors were weighed. He reported: 1) Allyliminothiadiazoline exerted a marked inhibitory effect almost comparable to that of Nitromin. 2) 6-Allylaminoquinoline was also very effective. 3) The effects of allylaminobenzene and β-allylaminonaphthalene were considerable; they were comparable to that of Sarkomycin.

In these experiments, the inhibitory effects of allyl-substituted compounds on tissue metabolism, not of Ehrlich solid tumor but of Yoshida ascites sarcoma cells, were studied. It may be unreasonable to compare the effect on tissue metabolism of Yoshida ascites sarcoma cells *in vitro* with the inhibitory effect on the growth of Ehrlich solid carcinoma. However, the inhibitory effects of a drug on the respiration and the glycolysis of Yoshida ascites sarcoma, Ehrlich ascites carcinoma, and rat ascites hepatoma cells are about the same (*cf.* table 2). Therefore, we believe that the comparison of inhibitory effects of drugs on the metabolism of Yoshida ascites sarcoma cells with growth inhibitory effects on Ehrlich solid carcinoma has some value.

It has been reported by Dr. Endo (*11*) that allyliminothiadiazoline and 6-allylaminoquinoline have a marked inhibitory effect on the growth of the solid form of Ehrlich carcinoma. In this study, these two compounds had a moderate inhibitory effect on the QO_2 and $Q_M^{N_2}$ of Yoshida ascites sarcoma cells *in vitro*. On the other hand, β-allylaminonaphthalene and α-allylaminonaphthalene inhibited the QO_2 and $Q_M^{N_2}$ strongly, though the former had a moderate tumor-inhibitory effect and the latter had a slight tumor-inhibitory effect in Endo's experiments. Allylaminobenzene, which had a moderate tumor-inhibitory effect, inhibited the respiration of Yoshida sarcoma cells moderately. The other allyl derivatives had only doubtful inhibitory effects on the tumor growth in Endo's studies. In our experiments, these compounds were without inhibitory effect on the anaerobic glycolysis of Yoshida ascites sarcoma cells, though some of them moderately inhibited the respiration.

Summary

Effects of 9 well-known antitumor compounds, 14 allyl compounds, and X irradiation on the respiration and glycolysis of ascites-tumor cells were investigated and compared with the growth-inhibitory effects on Ehrlich solid tumor. The present *in vitro* study indicated that all the

antitumor agents used do not show the same effect on the glycolysis and respiration of ascites-tumor cells. Inhibition, stimulation, and no effect were observed in both glycolysis and respiration. Thus, there was no general correlation between the effect of a compound on tissue metabolism and its effect on tumor growth.

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Variability of the *In Vitro* Sensitivity of Human Leukemic Lymphocytes to X Rays and Chemotherapeutic Agents[1, 2]

ROBERT SCHREK, IRVING A. FRIEDMAN,[3] *and* STANLEY L. LEITHOLD,[4] *Tumor Research Laboratory, Research Service and Hematology Section, Medical Service, Veterans Administration Hospital, Hines, Illinois*

The *in vitro* radiosensitivity of lymphocytes of the blood of patients with chronic lymphocytic leukemia was studied previously by the method of unstained cell counts (*1*), and by a slide-chamber method (*2*). A surprising variability was found from patient-to-patient, and in 2 out of 17 patients studied the blood lymphocytes were completely resistant to 1000 r *in vitro*.

The slide-chamber method has recently been improved (*3*) and has been used in the present study to measure quantitatively the sensitivity of leukemic lymphocytes to X rays and radiomimetic chemicals.

Summary of patients.—All the patients with a diagnosis of chronic lymphocytic leukemia or lymphosarcoma with leukemia who were in the Veterans Administration Hospital, Hines, Illinois, and the Cook County Hospital, Chicago, Illinois, during the periods from October 15 to December 4, 1956, and from May 8 to August 1, 1957, were used. This comprised 31 patients as listed in table 1.

Methods.—The methods have been described in detail in a previous publication (*3*). The leukocytes isolated from the blood of leukemic patients were suspended in equal parts of normal human serum and TC199. The suspensions were placed in special slide chambers and incubated at 37° C. Daily counts of viable lymphocytes were made by means of an inverted phase microscope. Before incubation, few cells (less than 2 percent) appeared to be nonviable. The percentage of viable lymphocytes after incubation was calculated with the original viable cell count as 100 percent. The data were summarized by survival curves and by 10 percent survival time. No formal experiments were done to determine the experimental error of the 10 percent survival-time measurement, but it is considered to be 20 percent.

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[3] Chicago Medical School, Chicago, Illinois.

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TABLE 1.—*Summary of clinical histories and sensitivity tests of patients with chronic lymphocytic leukemia or lymphosarcoma with leukemia*

Code No.*	Date of test	Diagnosis†	Clinical history			Treatment prior to test‖		Control percent survival (*2 days*)	Results of tests: 10 percent survival time			
			Sex, age, race‡	WBC (*cells/μml.*) §	Lymphocytes (%)	Date	Type		Control	X rays (*1000 r*)	HN_2 (*3 μg./ml.*)	TEM (*0.3 μg./ml.*)
H93a	10/29/56	CLL	M62W	120	91	1/12–2/24/53	X	85	5+	3. 3	2. 9	1. 83
						7/13–9/13/53	X					
b	11/2/56			129	96	10/30–11/1/56	X	80	4+	3. 6		
C95a	10/15/56	LSa	M73N	320	96	None			12+	10+		
b	10/31/56			208	96	None		100	9+	7+	7+	6. 3
C109	11/7/56	LSa	M50N	30. 6	96	2/29–5/9/56	TEM	100	8+	3. 1		
						5/17–6/1/56	X					
						8/15–9/5/56	TEM					
C117a	11/14/56	CLL	M86W	18	76	None		90	7+	0. 50		
b	5/29/57			20	75	None		78	10. 9	1. 31		
C118a	11/14/56	LSa	F55N	200	95	9/'51–9/'55	X	100	4+	5. 0		
						4/'56	HN_2-C					
						9/'56	TEM					
						9/27–10/21/56	CB1348					
						10/19–10/21/56	HN_2-C					
b	11/21/56			53		None		85	10. 2	4. 7		
c	11/28/56			65	89	None		100	10+	5. 7	6. 0	
H132	11/19/56	LSa	M41W	141	80	12/10/52–2/18/53	X	95	7+	1. 70	3. 5	
H134	11/21/56	LSa	M65W	96	98	None		85	9. 9	1. 20		
C136	11/21/56	LSa	F61N	19. 3	90	11/14–11/20/56	TEM	100	6+	3. 4		
C137	11/21/56	CLL	M58N	47. 4	71	11/'55	X	90	7+	1. 15		
C138a	11/21/56	CLL	M65W	31. 8	90	7/27–9/5/56	TEM-C	100	5+	2. 10		
b	5/15/57			28	91	1/10–1/16/57	X	88	11. 8	2. 75		
H146a	11/27/56	CLL	M65W	90	97	7/27–8/31/49	X	86	6. 0	0. 65		
						10/26–11/17/53	X					
b	11/30/56			58	89	None		96	5. 2	0. 70	1. 21	
c	12/4/56			106		12/3/56	X	65	4+	0. 75		
H422	5/8/57	CLL	M71N	7	88	9/'51	X	65	14. 2	1. 60		
						3/'53	X					
						5/2–6/17/55	X					
						3/11–5/6/57	X					

C423	5/8/57	LSa	F60N	58	56	4/17–5/8/57	X	91	8.6	1.53	3.1	
C424a	5/8/57	LSa	M57N	15	65	4/1–4/15/57	X	45	6.2	0.88	1.45	
						4/20–5/7/57	TEM-C					
b	5/22/57			22	79	5/8–5/21/57	TEM	35	3.0			0.96
H432	5/13/57	CLL	M65W	134	96	3/14–4/18/56	X	77	10.5	3.12	1.38	
						5/12/57	HN_2					
C461	5/22/57	CLL	M64W	56	74	7/25–8/26/55	X	95	9.6	1.35		2.0
H473	5/28/57	LSa	M40W	4.1	34	12/14/56–2/5/57	HN_2	70	10.7	1.95	1.95	
						12/24–12/27/56	X					
C475a	5/29/57	CLL	M73W	13	30	None		45	9.6	1.56		
b	6/26/57			8.9		None		24	6.8	0.84		
C476	5/29/57	CLL	F73N	134	97	5/'53	ACTH	75	13.1	2.06		
						1/'56	C, ACTH, TEM					
						3/20–5/24/57	C, TEM					
C477	6/3/57	LSa	M40W	15	72	11/7–12/17/47	X	50	12.0	1.28		1.29
						6/22–7/15/49	X					
C491	6/6/57	LSa	M29W	11	65	6/3–6/5/57	ACTH, HN_2	15	3.8	0.75		
H492a	6/6/57	LSa	M66W	844	97	None		37	10.1	2.00		
b	6/20/57			622	80	6/6–6/9/57	HN_2	67	14		8.0	
H499	6/10/57	CLL	M60N	111	82	None		78	13	0.74	3.28	
H511	6/24/57	LSa	M43W	39	97	1/19/56	ACTH	36	11	1.0		
						1/23–1/24/56	HN_2					
						1/31–2/17/56	X					
H513	6/25/57	CLL	M61W	62	92	4/14–6/18/55	X	23	6.2	1.15		
						3/28–4/24/56	X, ACTH					
						12/17/56–1/30/57	X					
C521a	6/26/57	CLL	M73W	208	90	None		67	13	0.34		
b	7/10/57			170	92	None		27	4.7		2.30	
C540	7/3/57	CLL	F65N	14.0	67	None		57	9.8	1.44	1.38	
C570a	7/17/57	CLL	M82W	268	95	None		78	11.8	1.42	3.7	
H577	7/22/57	LSa	M48W	95	99	9/24–11/22/49	X, HN_2, P^{32}	65	9.3	1.18	1.51	
						11/31/56–1/30/57	X					
H582	7/24/57	LSa	M65W	113	92	None		60	5.8	1.20	1.38	
H596	7/30/57	LSa	M67W	15	19	None		90	11	9.9	9.9	4.4

*Code number indicates the hospital (H=Hines Hospital and C=Cook County Hospital), the number of the patient, and the test number (a, b, and c=first, second, and third test on a patient).

†CLL=Chronic lymphocytic leukemia; LSa=Lymphosarcoma with leukemia.

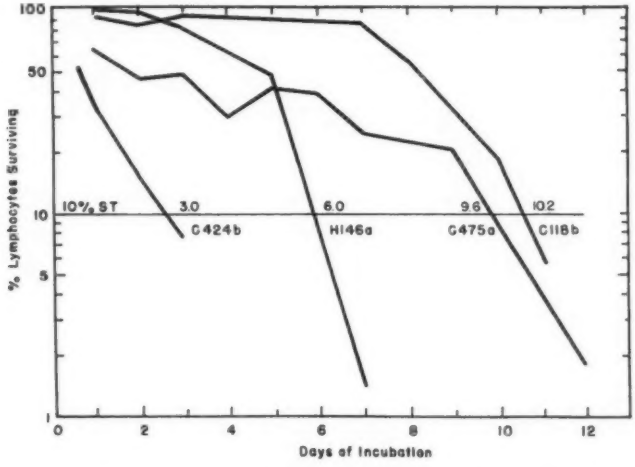
‡M = male, F = female, N = Negro, W = white.

§To convert to cells per mm^3, multiply count by 1,000.

||*X* = X-ray, TEM = triethylenemelamine, HN_2 = nitrogen mustard, C = cortisone, CB 1348 = Chlorambucil, ACTH = adrenocorticotropic hormone.

Before incubation some of the slide chambers were irradiated with a 100 kv. X-ray machine, 5 ma., 0.5 mm. equivalent filter, and HVL of 1.3 mm. Al. To administer 1000 to 4000 r, the distance was 10.5 cm. and the rate, 670 r per minute. For 100 r, the distance was increased to 31.5 cm. and the rate was 67 r per minute. The dosage of 10 r or less was given at the rate of 7.5 r per minute, the distance was 31.5 cm., the filter was 0.25 mm. Cu and 0.5 mm. equivalent Al, and the HVL was 0.94 mm. Al.

Survival of untreated leukemic lymphocytes.—Text-figure 1 shows the lymphocyte survival curves of 4 different patients. It is seen that the viable cells of C118b showed no appreciable decrease in number for about 7 days and that 10 percent of the lymphocytes survived 10.2 days. This type of curve was obtained for 19 of 31 patients with 10 percent survival time varying from 9 to 14 days, as indicated in table 1. The cells of patient C475a also had a long 10 percent survival time of 9.6 days, but during the first 2 days of incubation, the percentage of surviving cells decreased rapidly to 50 percent of the original count and remained on a plateau for 2 or 3 days. It is not known whether the initial decrease is biologically significant or an artifact. The cells of about 7 patients gave this cell-survival curve which is characterized in table 1 by a long 10 percent survival time (8 days or more) and by a relatively short 2-day percentage survival (20 to 60%). A different survival curve was found for H146a (text-fig. 1) with a 10 percent survival time of only 6.0 days. An even shorter 10 percent survival time was obtained for C424b (3.0 days as shown in text-fig. 1) and for C491 (3.8 days, table 1). The limited data



TEXT-FIGURE 1.—Survival curves of blood lymphocytes from 4 patients with chronic lymphocytic leukemia. The ordinates indicate the percentage of lymphocytes that remain viable in the slide chambers at 37° C. with 100 percent representing the number of viable lymphocytes during the first few hours of incubation. Three of the patients, but not C475a, had received clinical treatment prior to the *in vitro* test, as indicated in table 1.

so far available (table 1) show no clear correlation between short lymphocyte survival time and previous thereapy.

It is shown in text-figure 1 that the number of viable cells of patient C424b rapidly decreased within 3 days. Microscopic examination of a fresh slide preparation during the first hour of incubation showed that many of the cells were undergoing lobulation, intranuclear vacuolization, and death, which were phenomena rarely encountered in other fresh specimens of normal or leukemic blood. In view of these findings, it is probable that patient C424b had a relatively high percentage of dead cells in his blood. Similarly, Petrakis *et al.* (*4*) found that freshly drawn blood, particularly from leukemic patients, frequently contained many dead cells as indicated by the method of unstained cell counts.

In at least 20 of 31 patients with chronic lymphocytic leukemia, the blood lymphocytes had, in the slide preparations, a 10 percent survival time of 9 to 14 days. In previous work (*1*), the untreated cells of 5 patients with chronic lymphocytic leukemia had an average 10 percent survival time of 7 days when the cells were incubated and shaken in a test tube and the viable cell counts were made by the unstained cell method. It may be concluded that in most patients leukemic lymphocytes showed good survival *in vitro* whether the cells were incubated in a slide chamber or in a test tube, and whether the cells were considered viable by morphologic characterization or by eosin stainability.

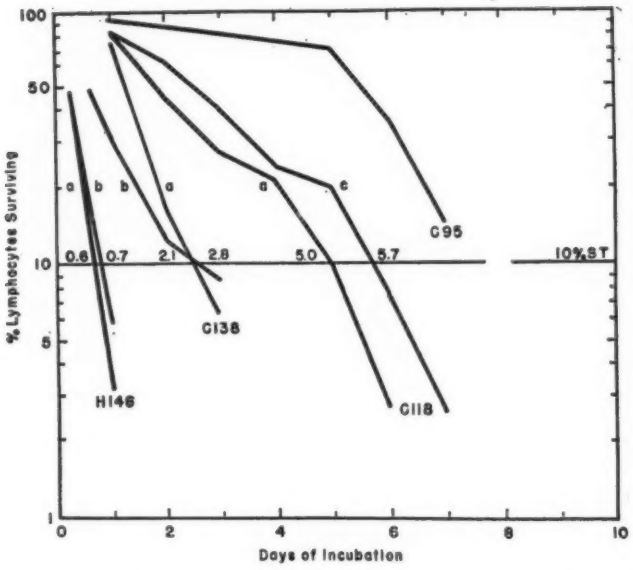
Effect of X Rays

Variability of Sensitivity Tests on Different Patients

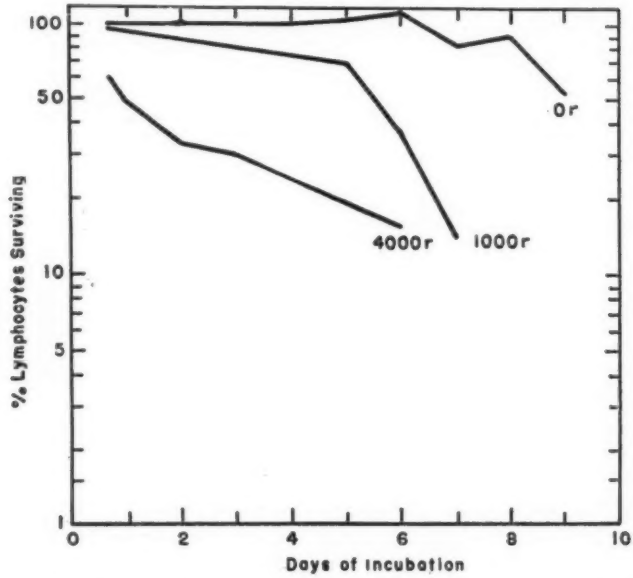
The cellular suspensions in the slide chambers were irradiated with 1000 r and incubated at 37° C. Text-figure 2 presents typical survival curves for the irradiated blood lymphocytes of 4 patients with chronic lymphocytic leukemia. The figure shows that the curves for the 4 patients differed from each other, and that the 10 percent survival time of the irradiated cells varied from 0.6 days for patient H146 to more than 7 days for C95. The survival curves for the nonirradiated cells of patients C118 and H146 are presented in text-figure 1.

In a previous study with unstained cell counts (*1*), it was found that the 10 percent survival times of the white blood cells of 4 patients varied from 1.1 to 3.6 days and that the cells of the 5th patient showed no appreciable reaction to 1000 r. According to the 2 *in vitro* methods, patients with chronic lymphocytic leukemia varied considerably in the sensitivity of their blood cells to 1000 r.

The resistance of the cells of C95 seemed of particular interest. Text-figure 3 compares the survival curves of untreated cells and cells irradiated with 1000 and 4000 r. It is seen that 1000 r produced little or no effect on the survival of the cells during 5 days of incubation. Irradiation with 4000 r caused a slow decrease in the survival curve though 10 percent of the cells resisted the irradiation for 6 days. Although the cells of C95 were morphologically similar to those of many other patients in the series, they differed in their high resistance to X rays.

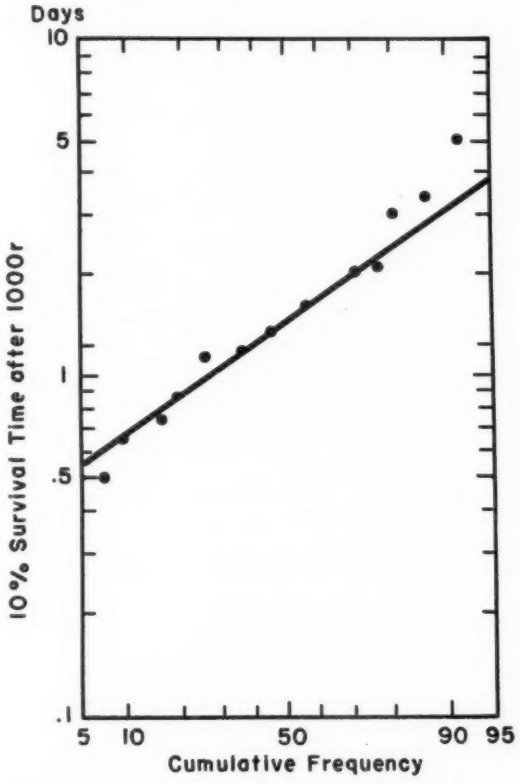


TEXT-FIGURE 2.—Survival curves of irradiated (1000 r) blood lymphocytes from 4 patients with chronic lymphocytic leukemia. Repeat tests were made on 3 patients. The sensitivity of the cells to X rays is represented by the 10 percent survival time which is determined from the survival curves.



TEXT-FIGURE 3.—Survival curves of untreated and of irradiated blood lymphocytes from patient C95.

The cells of 31 patients with chronic lymphocytic leukemia were tested for sensitivity to 1000 r, and the data are given in table 1. Text-figure 4 presents, on logarithmic probability paper, a frequency distribution curve of the 10 percent ST-1000 r obtained on the first test of these patients. It is seen that the frequency distribution can be represented by a straight line. The linearity of the curve indicates a "normal" frequency distribution. As described in a previous paper (*5*), a linear logarithmic



TEXT-FIGURE 4. Frequency distribution of the 10 percent ST-1000 r of blood lymphocytes from 31 patients with chronic lymphocytic leukemia or lymphosarcoma with leukemia. The abscissa indicates cumulative frequency in probability units and the ordinates show the logarithm of the 10 percent survival time.

distribution is best characterized by the geometric mean and standard deviation, which were found in this case to be 1.61 days and 2.08, respectively.

Table 2 presents a comparison of the sensitivity tests on 15 patients with chronic lymphocytic leukemia and 16 with lymphosarcoma in leukemic phase. The lymphosarcoma group had relatively high arithmetic and geometric means and included all 4 patients with highly resistant

TABLE 2.—*Summary of sensitivity tests on blood lymphocytes from leukemic patients to X rays, nitrogen mustard, and triethylenemelamine*

Comparison of:	X rays: 10 percent ST-1000 r			HN_2: 10 percent ST-3μg./ml.	TEM: 10 percent ST-0.3μg./ml.
	All patients	Chronic lymphocytic leukemia	Lymphosarcoma with leukemia		
Number of patients	31	15	16	17	5
Geometric mean (*days*)	1. 61	1. 26	2. 02	2. 80	2. 23
Geometric standard deviation	2. 08	1. 85	2. 18	1. 94	
Arithmetic mean (*days*)	2. 18	1. 50	2. 88	3. 53	2. 79
Arithmetic standard deviation (*days*)	2. 16	0. 84	2. 88	2. 56	
Coefficient of variation	99%	63%	100%	72%	
Median (*days*)	1. 4	1. 4	1. 4	2. 9	1. 9
Range (*days*)	0. 3–10+	0. 3–3. 3	0. 8–10+	1. 2–9. 9	1. 0–6. 3

cells (10% ST-1000 r more than 3.3 days). The observed differences, however, are not statistically significant.

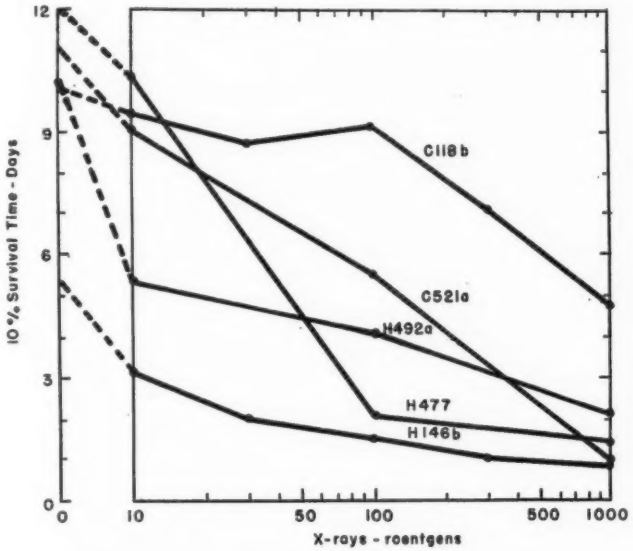
Variability of Sensitivity Tests on the Same Patient at Different Times

Repeat tests were done on 7 patients, as shown in table 1, to determine the reliability of the *in vitro* test and the variability of the patient. Text-figure 2 presents duplicate survival curves on 3 patients. It is seen that repeat tests on the patients gave approximately similar survival curves for 1000 r-treated cells. In table 1, the findings on the second and third tests of 6 patients agreed fairly well with the results obtained on the first. Most of the repeat tests were done within 2 weeks after the first test. C138 had a repeat test 6 months after the first with a slight increase in resistance of the cells (2.10 and 2.75 days). C117 also had a repeat test in 6 months and the second result (1.31 days) was also higher than the first (0.5 days). So far as they go, the data indicate that the lymphocyte radiosensitivity in any one patient remained fairly constant.

Variability of Lymphocytes From the Same Patient

It was not possible to study directly the variability of the radiosensitivity of the leukemic lymphocytes from the same patient. The variability could be inferred by testing the cells with several different dosages of X rays, usually 1000, 100, and 10 r.

The 10 percent ST after different doses is shown in text-figure 5 for 5 patients. It is seen that the curves differ from each other in position, in slope, and in shape. Evidently the 10 percent survival time is variable for different patients not only after 1000 r but also after 100 and 10 r. Patients H492a and C521a had approximately the same sensitivity to 1000 r but differed in their sensitivity to 10 r. The variable slopes and shapes of the dose-effect curves show that the radiosensitivity of the cells of a patient with chronic lymphocytic leukemia cannot be characterized completely by testing with a single dose; it is necessary to study the effects of at least 10, 100, and 1000 r.

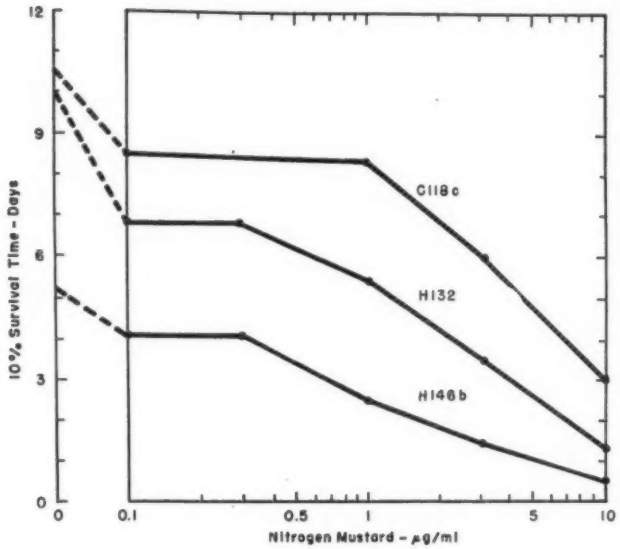


TEXT-FIGURE 5.—The dose-effect curves showing the effect of 10 to 1000 r on the 10 percent survival times of blood lymphocytes from 5 patients with chronic lymphocytic leukemia.

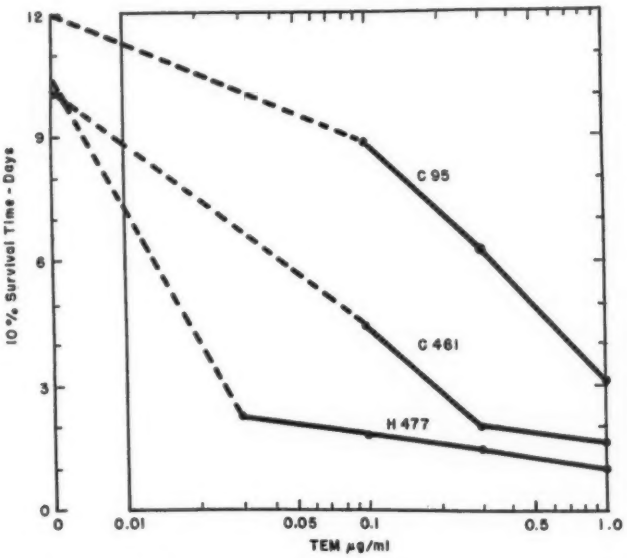
Effect of Radiomimetic Drugs

The radiomimetic drugs studied were triethylenemelamine (TEM) and nitrogen mustard HN_2 [methyl-bis(2-chloroethyl)amine hydrochloride]. The chemicals in suitable dilutions in distilled water were added to the cellular suspension just before slide preparations were made. Some dose-effect curves obtained are shown in text-figures 6 and 7. It is seen in the figures and in table 2 that the cells of different patients varied considerably in their sensitivity to the radiomimetic agents.

The cells of 19 patients were tested with 2 or 3 reagents, as shown in table 1. Text-figure 8 compares the sensitivity of the leukemic cells of these patients to 1000 r of X rays, 3 μg. per ml. of HN_2 and 0.3 μg. per ml. of TEM. These dosages were selected because they produced approximately equivalent effects on lymphocytes from normal blood. The ab-

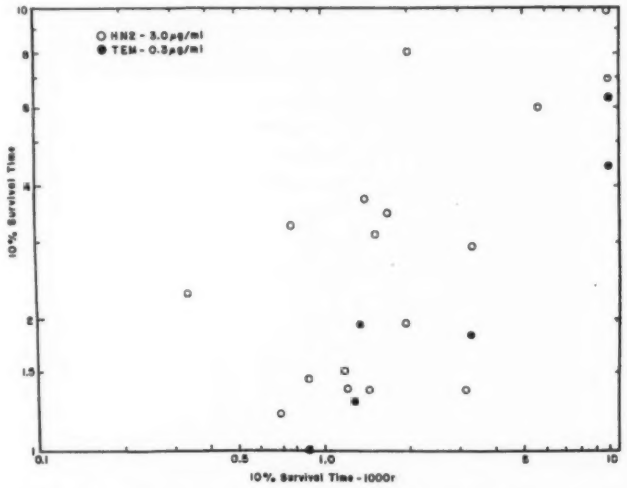


TEXT-FIGURE 6.—Dose-effect curves showing the effect of nitrogen mustard on the blood lymphocytes of 3 patients with chronic lymphocytic leukemia.



TEXT-FIGURE 7.—Dose-effect curves showing the effect of triethylenemelamine on the blood lymphocytes of 3 patients with chronic lymphocytic leukemia.

scissa gives the 10 percent ST-1000 r in logarithmic units and the ordinates, the 10 percent survival time after HN_2 or TEM. The correlation coefficient between the log (10% ST-1000 r) and the log (10% ST-3 μg./ml. of HN_2) is 0.64 $\pm$ 0.14 standard deviation. There is then a significant correlation between the sensitivity of the cells to X rays and to nitrogen mustard.



TEXT-FIGURE 8.—Scatter diagram to show the relationship between the sensitivity of the cells of 19 patients to X rays and to radiomimetic agents. Each point represents the sensitivity tests on leukemic cells of a patient to X rays (10% ST-1000 r) and either to HN_2 (10% ST-3 μg./ml.) or to TEM (10% ST-0.3 μg./ml.).

Reactions of Nonleukemic Lymphocytes

Parallel studies on lymphocytes from normal individuals and nonleukemic patients are in progress, and a preliminary analysis of the data is given in table 3. It is seen that the nonleukemic lymphocytes survived satisfactorily in the slide chambers (10% survival time, 6 to 14 days). The normal or nonleukemic lymphocytes show relatively slight variability in sensitivity to X rays with a 10 percent ST-1000 r of 1.1 to 2.2 days for 16 individuals. Similarly, the lymphocytes from nonleukemic individuals show only moderate variability to HN_2 (1.2 to 2.9 days) and to TEM (1.9 to 2.4 days). It seems that the lymphocytes of nonleukemic individuals show less variability in their reactions to the reagents than lymphocytes from leukemic patients.

Discussion

Variability of Patients with Chronic Lymphocytic Leukemia

According to the sensitivity tests in this study, some patients had blood lymphocytes that were killed by 10 r of X rays while other patients

had cells that resisted 100 or even 1000 r. The 10 percent survival time after 1000 r varied from 0.3 to 10 days for blood lymphocytes from different patients. The variability of the sensitivity test was high as indicated by the wide range, the large coefficient of variation (99%), and the corresponding high geometric standard deviation (2.08). Such high constants of variation are unusual in biologic frequency distributions. Similar high variability was observed in the sensitivity tests with nitrogen mustard and triethylenemelamine. These findings lead to the conclusion that patients with chronic lymphocytic leukemia or lymphosarcoma with leukemia are highly variable in the sensitivity of their blood lymphocytes to reagents.

Is the finding of the variability of leukemia patients reasonable and valid? First it is necessary to consider the reliability of the method. It has been seen in this study that repeat tests on the same patients gave consistent and reproducible results. In parallel studies, sensitivity tests

TABLE 3.—*Comparison of the reactions of blood lymphocytes from nonleukemic individuals and from patients with chronic lymphocytic leukemia or lymphosarcoma with leukemia*

	10 percent survival time	
	Nonleukemic lymphocytes (*days*)	Leukemic lymphocytes (*days*)
Range		
Untreated cells	6–14 (26)*	3–14 (32)
Cells treated with:		
X rays: 1000 r	1.1–2.2 (16)	0.3–10 (31)
HN_2: 3 μg./ml	1.2–2.9 (7)	1.2–9.9 (17)
TEM: 0.3 μg./ml	1.9–2.4 (4)	1.1–6.3 (5)
Geometric mean		
Cells treated with:		
X rays: 1000 r	1. 68	1. 61
HN_2: 3 μg./ml	1. 97	2. 80
TEM: 0.3 μg./ml	2. 20	2. 23

*Numbers in parentheses indicate the numbers of patients tested.

on blood lymphocytes of nonleukemic individuals showed relatively slight variability (table 3). These two findings suggest that the observed variability of the sensitivity tests for different leukemic patients is biologically significant and not due to the method.

The present findings may be compared with reports on the variability of animal and human tumors. Goldfeder and Cameron (*6*) found that two mammary adenocarcinomas of the mouse, though histologically similar, differed in the *in vitro* sensitivity of their cells to X rays. The cells of one tumor in tissue culture was sensitive to 20,000 r while the cells of the other tumor resisted 100,000 r. Kirschbaum *et al.* (*7*) observed differences in the *in vivo* sensitivity of transplantable lymphoid leukemias of the mouse to chemotherapeutic agents. Karnofsky (*8*) commented on the diversity of therapeutic response in histologically similar cancers and leukemias of man. The present findings agree with these studies in showing that tumors and leukemias with the same morphologic classi-

fication do not necessarily have the same physiologic or pharmacologic characteristics.

According to the sensitivity tests, some patients had cells that were sensitive to 1000 r but not to 10 r. *A priori*, these patients might be expected to respond to high dosages of X rays administered to lymph nodes or spleen but not to low dosages of spray X rays. Studies on the clinical response of patients to therapy are in progress.

Radiosensitivity and Maturity of Lymphocytes

The quantitative data that has been obtained for normal and leukemic lymphocytes may aid in understanding the radiosensitivity of the lymphocyte. Irradiation has been shown to kill the normal lymphocyte whether it is derived from blood, thymus, or lymph node (*9*). On the other hand, the immature lymphocyte or lymphoblast obtained from patients with acute lymphocytic leukemia has been found to be resistant to X rays (*2*). The same resistance has been observed (*10*) in the lymphoblast of transplantable rat tumors (Bagg lymphosarcoma, reticulum-cell type, and the Lewis lymphosarcoma). So far only the malignant lymphoblast has been available for testing. These findings suggest the hypothesis that sensitivity to X rays depends on the maturity of the cell.

Summary

A slide-chamber method was used to test 31 patients with chronic lymphocytic leukemia for the sensitivity of their blood lymphocytes to X rays and radiomimetic drugs *in vitro*. The results of the radiosensitivity tests were expressed as 10 percent ST-1000 r, *i.e.*, the 10 percent survival time of the cells after treatment with 1000 r. Repeat tests on the same patients showed good reproducibility of the determinations of 10 percent ST-1000 r. The patients differed in the sensitivity of their blood lymphocytes to X rays with the 10 percent ST-1000 r varying from 0.3 to 10+ days. The effect of 10 to 1000 r of X rays on the 10 percent survival time was represented by dose-effect curves, which were found to vary for different patients with respect to position, shape, and slope. The lymphocytes of the patients also varied in their sensitivity to nitrogen mustard (HN_2) and triethylenemelamine (TEM). Nineteen patients were tested with 2 or 3 reagents. A significant correlation was found between the sensitivity of a patient's lymphocytes to X rays and to nitrogen mustard.

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Immunological Characteristics of Radiation Chimaeras [1]

P. L. T. Ilbery,[2] P. C. Koller,[3] *and* J. F. Loutit,
Medical Research Council, Radiobiological Research Unit, Atomic Energy Research Establishment, Harwell, and Chester Beatty Research Institute, London, England

It has been established that hematopoietic tissues of mice are destroyed after exposure to a lethal dose of total-body X radiation and that damage could be repaired by injecting bone marrow from normal, nonirradiated mice (*1*). The recipient individuals, which survive the lethal radiation dose due to the restoration of hematopoietic activity by recolonization of the marrow, can be defined as "chimaeras"; they contain functioning donor cells which may differ genetically from those of the X-rayed host (*2–4*). The symbiotic coexistence of genetically different tissues in radiation chimaeras represents an interesting aspect of cellular adaptation. An analysis of immunological response in these genetically mosaic individuals would be expected to add to the understanding of the phenomenon of "acquired tissue tolerance."

The immunological behavior of radiation-induced mouse chimaeras has been investigated by Barnes *et al.* (*5*) by the method of tumor transplantation. These preliminary experiments indicated that the "neutral state" of chimaeras, presumably due to suppression of the immune mechanism of the host by radiation, is of short duration. By challenging the chimaeras with tumors, it was noted that the age of chimaeras, *i.e.*, the length of interval between irradiation and challenge with tumor, influenced the fate of tumor graft. The present investigation was planned to study this phenomenon in more detail and to explore the possibility of conferring the donor's immunological characteristics on the host by transferring cells from lymph glands. Billingham *et al.* (*6*, *7*) and Mitchison (*8*), using nonirradiated hosts, obtained evidence that the immunologically activated tissue of the immunized donor continues to function after transfer. It was hoped that more might be revealed about the nature of such adopted immunity by a study of radiation-induced mouse chimaeras.

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[2] Holder of a New South Wales, Australia, Cancer Research Fellowship. *Present address:* Department of Preventive Medicine, University of Sidney, New South Wales, Australia.

[3] The author is greatly indebted to the Atomic Energy Research Establishment for a grant and the Medical Research Council for facilities to work in the Radiobiological Research Unit, Harwell. *Present address:* Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S. W. 3, England.

Materials and Methods

Mouse Strains and Transplantable Tumors

Mice of inbred strains A, CBA, C3H, C57BL, and F_1 crosses between these strains were used as host or donor. The mice used in other hybrid combinations were:

The highly inbred, wild type, agouti (A^wA^w), strain 101 established by L. C. Dunn, of Columbia University, New York; PCT stock carrying 7 homozygous recessive genes (nonagouti: *aa;* brown: *bb;* chinchilla: *chch;* dilute: *dd;* short ear: *sese;* spotted: *ss;* pink eye: *pp*) brought together by W. L. Russell, Oak Ridge; and T6, which is a stock with a translocation involving the third linkage group and, in the homozygous state, is characterized cytologically by a pair of extremely small acrocentric chromosomes (*3*).

These stocks have been used in the following combinations: R, AH, T6 × (C3H × 101)F_1.

R symbolizes the offspring of the cross between (C3H × 101)F_1 and PCT.
AH symbolizes the offspring of a cross between strain A and (C3H × 101)F_1.

Mice used in the experiments were bred in the Radiobiological Research Unit, Harwell, with the exception of the (A × C57BL)F_1 mice, which were obtained from the Chester Beatty Research Institute, London.

Only males between 90 to 120 days old at the time of radiation were used as hosts.

The following 6 transplantable tumors were available for grafting:

1) Sarcoma I—strain A tumor: This tumor will also grow progressively and kill some CBA, C3H, and R mice; the incidence, however, is never more than 10 percent.

2) *MT03*—well-differentiated mammary-gland tumor that originated in strain A mice of the Chester Beatty Research Institute: The tumor is highly specific and slow growing; 1×10^6 cells injected subcutaneously kill the host at 25 to 30 days.

3) *MT13*—mammary-gland tumor that is anaplastic without differentiation, and originated in a C3H mouse of the Chester Beatty Research Institute. The tumor is very specific and grows progressively only in C3H. Subcutaneous injection of 1×10^6 cells kill the host at 25 to 30 days.

4) *BP8*—benzopyrene-induced sarcoma in a C3H mouse (Dr. J. Craigie, of Imperial Cancer Research Fund, London): The tumor grows progressively only in C3H and CBA mice and not in other strains; 1×10^6 cells injected subcutaneously kill at 12 to 14 days in C3H; and 30 to 35 days in CBA.

5) *151/1*—lymphoma induced by X ray in a CBA mouse (Dr. R. Mole, Harwell): The tumor is strain-specific and kills in 21 to 28 days when 1×10^6 cells are injected subcutaneously.

6) *138/2*—lymphoma induced by X ray in C57BL mouse (Dr. J. F. Loutit). It is highly strain-specific; 1×10^6 cells injected subcutaneously kill the host at 21 to 28 days.

Radiation

Five mice, or occasionally 6, were kept together and exposed to X irradiation in their breeding boxes (*9*). The following doses were given to mice of the various stocks:

Dose(*r*)	Strains and hybrids
1000	R, AH
950	C3H, (A × CBA)F_1, CBA*
850	A, (A × C57BL)F_1
800	C57BL

*In some experiments 1000 r.

The radiation was delivered as a single dose given at the rate of 44 r per minute with the following factors: 240 kv. constant potential, 15 ma., HVL = 1.2 mm. Cu.

Donor Tissue

The following donor material was used in our experiments:

1) *Bone marrow:* Marrow was taken from 2 femurs and diluted with 1 ml. physiological saline solution. Each irradiated mouse was given 0.2 ml. of this marrow suspension by intravenous injection into the tail vein. The number of cells in this amount is between 2×10^6 and 5×10^6. The age of the donor was about 3 or 4 months, and the sex was usually male.

2) *Spleen:* A 1-week-old baby mouse was sacrificed, its spleen macerated, and the cell suspension diluted with 2 ml. of fresh rabbit serum (*10*). Each mouse was given 0.4 ml. (about 3×10^6 cells) by intravenous injection.

3) *Lymph glands:* Six glands (2 axillary, 2 brachial, and 2 inguinal) were taken from the same animal that supplied the marrow. The glands were minced in 0.5 ml. saline and centrifuged through a modified Seitz bacterial filter. Nylon of 60 to 80μ mesh was used to retain the connective-tissue debris. The filtrate that contained about 3 or 4×10^6 cells was added to 0.5 ml. saline suspension of bone marrow. When immunized donors were used, only one lymph node, usually the inguinal, was taken to supply the cells for transfer. The node was about 4 or 5 times larger in the immunized than in neutral mice and yielded a greater number of cells; thus one was sufficient for the purpose. The interval between irradiation of the host and administration of the cell suspension from the donor was usually not more than 2 to 4 hours.

Definition of Radiation Chimaeras

The chimaeras are mice that were exposed to LD100 total-body X irradiation and were given cell suspension of various tissues from normal donors. The origin of donor material determines the nature of chimaeras:

1) *Isologous chimaera:* When both the host (the X-rayed recipient in this case) and the donor are taken from the same subline of inbred strain, *e.g.*, CBA host and CBA donor; or both are mice from the first-generation cross between the same 2 inbred strains, *e.g.*, (A $\times$ C57BL)F_1 host and (A $\times$ C57BL)F_1 donor.

2) *Semi-isologous chimaera:* When mice of 2 inbred strains are used in combination with the F_1 hybrid in which those particular strains are represented; *e.g.*, (A $\times$ C57BL)F_1 host and A donor, or C57BL host and (A $\times$ C57BL)F_1 donor.

3) *"Syngenetic" chimaera:* When composed of more than 2 strains, and one or more of these is common to host and donor; *e.g.*, the [(C3H $\times$ 101)F_1 $\times$ A] host and C3H donor, or the [(C3H $\times$ 101)F_1 $\times$ PCT] host and donor.

4) *Homologous chimaera:* When composed of genetically different host and donor and may involve more than 2 strains, *e.g.*, CBA host and C3H donor or the [(C3H $\times$ 101)F_1 $\times$ PCT] host and CBA donor.

5) *Heterologous chimaera:* When composed of host and donor that belong to different species, *e.g.*, mouse host and rat donor. In the present investigation heterologous chimaeras were not used.

In the symbols used in text and tables, the host constitution is given as the first component and that of the donor as the second, *e.g.*, CBA/C3H means CBA host and C3H donor.

The "age of chimaeras," referred to in text and tables, is calculated from the date of irradiation and therapy.

Immunization

One aim of the present investigation was to transfer marrow and lymph-node tissues of immunized donors to the irradiated host in order to study

the immune responses of such chimaeras by challenging them with tumor grafts. For this purpose, 3- to 4-month-old male mice were immunized against foreign tumors by two subcutaneous injections of 1×10^6 cells. The immunized mice usually were used as donors 3 or 4 weeks after the second injection. In a few instances, immunized mice were used as hosts; R immunized against Sarcoma I.

Tumor Challenge

Challenge was carried out by injecting subcutaneously 1×10^6 tumor cells in 0.4 ml. saline into the flank of the chimaeras. As a control, the same number of tumor cells was injected into nonirradiated mice of the strain in which the tumor had originated. When the chimaeras were challenged subsequently, with one or more tumors of different strains, the injections were made alternately into left and right flanks. If there was doubt about the identity of the tumor growing in chimaeras, the tumors were excised and passaged by Bashford needle into mice of various strains to establish the identity of the tumor.

Results

Isologous Chimaeras

In order to estimate the influence that the lymphoid tissue, recolonized from the donor, has on the take and growth of a tumor graft, CBA/CBA isologous chimaeras were challenged with Sarcoma I, a strain A tumor. It has been found that Sarcoma I can kill in about 3 percent of the CBA subline carried in Harwell. The response of 36 CBA/CBA chimaeras, which have been challenged subcutaneously with 1×10^6 cells of Sarcoma I, is shown in table 1.

It can be seen that 7 out of 36 (19%) isologous chimaeras have taken and been killed by the tumor; *i.e.*, the incidence of such individuals is about 6 times higher than in the normal CBA. The nonresistance of CBA/CBA chimaeras one may attribute to impairment of the immune mechanism. It is known that radiation produces an immunologically neutral state in animals, and that the duration of this condition depends primarily on the radiation dose. Thus it has been observed that 500 r produces a neutral state in mice of at least 14 to 16 days' duration during which period they could be successfully grafted with tumors of foreign strains (*cf.* fig. 17). It may be argued, therefore, that in CBA mice which had been exposed to 950 to 1000 r, LD100 total-body X irradiation, the inactivation of the immune mechanism would be more effective and the period of neutral state would be longer. In our experiments, the lymphoid organs of the irradiated mice are repopulated by donor cells as a result of bone-marrow therapy (BM); therefore the restoration of the immune mechanism could be expected. The data, however, seem to indicate that the immune response of the chimaera was not fully restored at 46 days after receiving 950 r and this therapy, as shown by the fact that a high proportion (33%) had been killed by Sarcoma I. Data of table 1, on the

TABLE 1.—*Immune response of isologous chimaeras*

Host	Donor	Donor tissue	Number of chimaeras	Age of chimaeras at tumor challenge (*in days*)	Tumor	Chimaeras with tumor	
						Number	Percent
CBA control*	—	—	—	—	Sarcoma I	—	3
CBA	CBA	BM	12	46	Sarcoma I	4	33
CBA†	CBA	BM + LG	15	22	Sarcoma I	2	13
CBA	CBA—immune Sa. I	BM	4	38	Sarcoma I	1	25
CBA†	CBA—immune Sa. I	BM + LG	5	22	Sarcoma I	0	0
Total			36			7	19

**Note:* Data based on 33 CBA mice.

†CBA hosts were given 1000 r total-body radiation.

other hand, also suggest that the addition of lymph gland to bone marrow (BM + LG) as donor material reduced the frequency of nonresistant chimaeras; only 2 out of 15 or 13 percent succumbed to Sarcoma I. The reduction is even more notable in view of the higher dose of radiation given to the CBA host and the earlier date when the chimaeras were challenged with the tumor.

Table 1 also gives the results of a single experiment in which the irradiated CBA mice were given bone marrow or bone marrow and lymph tissue from CBA mice immunized against Sarcoma I. The only chimaera that failed to resist challenge with sarcoma I had received no lymphoid tissue with the bone-marrow therapy.

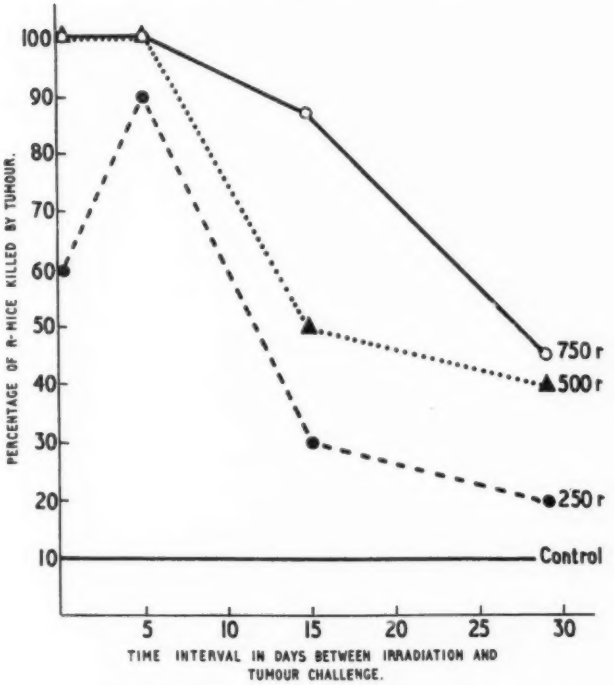
The influence of lymph gland on the immune response of CBA/CBA chimaeras has been shown by the behavior of the tumor graft itself. In "BM" chimaeras, *i.e.*, CBA/CBA chimaeras that were given only marrow tissue as donor material, the tumor Sarcoma I was manifest 7 days after injection of 1×10^6 cells; it appeared as a small subcutaneous nodule, the diameter of which was less than 0.5 cm. In the resistant individuals, these local tumor nodules became hard about 20 to 22 days after the chimaeras had been challenged. If the immune mechanism of the chimaeras was ineffective, the tumor increased in size; at 14 days it had a diameter of 0.5 to 0.8 cm. and was 2.5 to 3 cm. at the death of the animal at about 28 days. In "BM + LG" chimaeras, *i.e.*, X-rayed CBA's which were given bone marrow and lymph-gland material from a CBA donor, the tumor graft became visible and palpable at 7 days—about the same time as in the "BM" chimaera. In the resistant individuals it did not grow after 7 days but ulcerated, and at 21 days the site of the graft was indicated by a small, scaly plaque. When, however, the tumor was able to overcome the impaired or depressed resistance of the "BM + LG" chimaera, its progressive growth was slow since it took 42 days from implantation to attain the same size (2–3 cm. in diameter) as the graft in the 28-day-old "BM" chimaera.

While the data obtained with isologous chimaeras seem to indicate that the addition of lymph gland to the bone-marrow donor material influences the immune response, it did not affect the viability of the "BM + LG" chimaeras.

Syngenetic Chimaeras

R mice, a triple-cross hybrid, were also available for testing the immune response in radiation chimaeras. The LD100 total-body radiation dose is 1000 r for R mice and the survival rate of R/R syngenetic chimaeras is high. The incidence of R mice killed by subcutaneous grafting of Sarcoma I is about 10 percent.

Experiments have been carried out to determine the effect of sublethal doses of irradiation on the take and progressive growth of Sarcoma I in R mice. The data obtained are shown in table 2. It can be seen that the abrogation of immunity is influenced by the dose of irradiation and by the time interval which elapses between radiation and challenging the irradiated R's with Sarcoma I (text-fig. 1). The data show that about



TEXT-FIGURE 1.—Graph showing the abrogation of immunity in R mice: [(C3H × 101)F_1 × PCT], after various doses of X radiation.

TABLE 2.—*Effect of irradiation on immune response of R mice (Sarcoma I, subcutaneous 1×10^6 cells)*

Dose (*r*)	Time interval (*in days*) between radiation and challenge:							
	0		5		14–15		28–29	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
0	2/10*	20	1/5	20	1/20	5	3/20	15
250	6/10	60	9/10	90	6/20	30	4/20	20
500	10/10	100	10/10	100	10/20	50	8/20	40
750	10/10	100	6/6	100	15/17	88	8/18	44

*Number of mice killed by tumor/total number of mice challenged.

45 percent of R's succumbed to sarcoma I when challenged 28 or 29 days after having been irradiated with 750 r.

The response of R/R chimaeras, which had received 1000 r and were challenged with Sarcoma I, is shown in table 3. The age of chimaeras at the time of tumor challenge was 25, 28, and 38 days—in different experiments. Since 750 r suppressed immunity against Sarcoma I in 45 percent

TABLE 3.—*Immune response of "syngenetic" chimaeras [R = (C3H × 101)F_1 × PCT]*

Host	Donor	Donor tissue	Number of chimaeras	Age of chimaeras at tumor challenge (*in days*)	Tumor	Chimaeras with tumor	
						Number	Percent
R control*	—	—	—	—	Sarcoma I	7	12. 7
R	R	BM	18	28	Sarcoma I	13	70
R	R	BM + LG	6	25	Sarcoma I	0	—
R†	R immune Sa. I	BM + LG	12	25, 38	Sarcoma I	1	8(?)

*Data based on 55 R mice (*see* table 2).
†Six out of 12 R hosts were immunized against Sarcoma I 55 days before radiation + BM therapy.

of R mice that were challenged about the 30th day after irradiation, it could be expected that 1000 r would also yield a high number of nonresistant animals. The data show that only about 40 percent of R/R chimaeras were susceptible to Sarcoma I, which progressively grew and killed the chimaeras. The number of R/R chimaeras that succumbed to Sarcoma I was higher than in control R mice, indicating that donor marrow tissue was able to re-establish only partially the immune response.

When, however, the donor material was BM + LG, the immunity of R mice against sarcoma I was fully restored. Regression of the tumor was complete 24 to 26 days after the chimaeras had been challenged, and the small, scaly plaque at the site of the subcutaneous nodule disappeared. One chimaera was found to be nonresistant to this tumor, even though it received BM and LG from an R donor immunized against Sarcoma I. This represents an interesting exception. Having failed to show any sign of Sarcoma I after 53 days, this chimaera was challenged at 40-day intervals with 2 other tumors (151/1-CBA and BP8-C3H). Twenty-five days after the subcutaneous injection of 1×10^6 cells of BP8 tumor into the right flank, a rapidly growing nodule appeared, which was assumed to be BP8, the C3H tumor. When the chimaera had to be sacrificed 15 days later, owing to the very large and ulcerating tumor, the peculiar cytological appearance of the local mass and the widely spread metastatic growth in spleen, liver, and thymus, which was found at *post-mortem* examination, suggested the need for a transplantation test for identifying the tumor. Pieces of the local tumor mass were implanted by trocar into mice of strains A and C3H. The tumor graft grew in A mice; it failed in C3H indicating that it was Sarcoma I, which had been grafted on the R/R chimaera 165 days previously.

Homologous Chimaeras

In order to estimate the influence of donor material on the immune response of chimaeras, it was most important to use genetically different hosts and donors. This project, however, met serious difficulty owing to the high number of casualties among such chimaeras. It has been found that homologous bone marrow is able to restore the serious damage to the hematopoietic system after LD100 total-body X irradiation (*1*, *10*), yet many of the survivors suffer from "secondary radiation disease," from which they fail to recover (*11–14*). Such chimaeras, after partial recovery from the initial effects of the radiation, lose further weight in the 2nd month, become hunched, scruffy, and usually suffer from diarrhea.

During the present investigation two kinds of homologous chimaeras were made with CBA mice as the host and C3H or T6+ mice as donors. The survival rate and the immune response of these chimaeras are shown in table 4.

Survival rate.—It has been observed that our homologous chimaeras CBA/C3H or CBA/T6+, having developed "secondary disease," could survive for 8 weeks before dying from this illness. For this reason only those chimaeras which lived beyond 55 days after radiation and protection

TABLE 4.—*Survival rate and immune response of homologous chimaeras*

Host	Donor	Donor tissue	Number of chimaeras	Number of casualties and day of death	Survivors at 55 days		Tumor	Age of chimaera at tumor challenge (*in days*)	Chimaeras with tumors	
					Number	Percent			Number	Percent
CBA	C3H	BM	14	1 (5), 1 (38), 1 (42)	11	79	BP8 (C3H)	96	10	91
CBA	C3H	BM + LG	15	2 (6), 1 (12), 1 (15), 2 (54), 1 (55)	8	53	151/1 (CBA)	89	8	100
CBA	C3H—immune Sa. I	BM + LG	5	1 (30), 1 (38), 1 (39)	2	40	Sa. I (A)	38	2	100
CBA	C3H	Spleen	5	Nil	5	100	Sa. I (A)	96	3	60
CBA	C3H	Spleen	5	Nil	5	100	BP8 (C3H)	94	4	80
CBA	C3H—immune Sa. I	BM + LG	7	3 (6), 1 (7), 1 (8), 1 (43), 1 (44)	0	0	—	—	—	—
Total			61		31	50. 8				
CBA	T6+immune 151/1	BM	5	1 (36), 1 (40), 1 (41), 1 (50)	1	20	151/1 (CBA)	213	1	(100)
	T6+	Spleen	5	1 (56)	4	80	151/1 (CBA)	96	4	100
	T6+	Spleen	4	Nil	4	100	138/2 (C57BL)	96	0	0
Total			14		9	64				

were classified as survivors in table 4. About 50 percent of the CBA/C3H and 64 percent of the CBA/T6+ chimaeras survived this period.

Immune response.—The homologous chimaeras that survived or escaped "secondary disease" have been challenged with various tumors in order to see whether they were immunologically neutral or not and, in the latter case, whether the immunological characteristics were those of the host or donor. Though the numbers are small, the data obtained indicate two facts: (*a*) that these chimaeras were not neutral, as shown by the complete immunity of CBA/T6+ chimaeras against the 138/2 lymphoma of C57BL strain; (*b*) that the immune mechanism of the chimaeras was not controlled by the host alone; the high proportion of CBA/C3H chimaeras which were not resistant to Sarcoma I suggests that the combination of donor and host tissue affected the immune responses.

Semi-isologous Chimaeras

By using the first-generation cross between 2 inbred strains, either as host or donor in combination with one of the parental strains, the semi-isologous chimaeras which are obtained are most suitable for the study of factors affecting survival and immune response. The data obtained with semi-isologous chimaeras are given in table 5.

Survival rate.—The survival rate of chimaeras is somewhat difficult to estimate from table 5 owing to the fact that several individuals were challenged with tumor within the 55 days, the limit set as the last day of death from secondary disease. Some chimaeras died before this time limit and the cause of death might have been either the tumor or the radiation-induced secondary disease, or both. However, all the casualties have been examined *post mortem* and correction could be made to estimate the true survival rate of the various semi-isologous chimaeras. If a chimaera died between the 42nd and 55th day with tumor but without showing the symptoms of secondary disease, it was included among the 55-day survivals.

The corrected data of table 6 show that the number of surviving chimaeras is higher when the donor is the F_1 hybrid than when it is one of the parental strains. Another interesting fact is that the incidence of death among the "BM + LG" chimaeras is higher than in the "BM" chimaeras; the difference is clearly shown among the chimaeras with the F_1 constitution as the donor. In the favorable combination of "BM" chimaeras about 60 percent survived beyond 55 days, while in the "BM + LG" chimaeras only 23 percent survived. A similar trend has been observed in the survival rate of CBA/C3H chimaeras when comparing the efficiency of BM as against BM + LG (table 4).

Immune response.—The semi-isologous chimaeras have been challenged either with Sarcoma I or with 138/2-C57BL lymphoma, depending on the nature of donor and host. The strain A donor mice were immunized against 138/2 tumor and the C57BL donor mice against Sarcoma I. Though the number of survivors that could be tested for their immunological specificity is small, the data of table 5 seem to indicate that no effective

TABLE 5.—*Survival rate and immune response of semi-isologous chimaeras*

Expt. No.	Host	Donor	Donor tissue	Number of chimaeras	Number dead / Day of death	Age of chimaera at tumor challenge (*in days*)	Tumor	Tumor positive: Number challenged	Note
65	(A × C57BL)F$_1$	(A × C57BL)F$_1$	BM	5	1/51	—	—	—	Four alive 150 days after radiation and protection.
31	(A × C57BL)F$_1$	(A × C57BL)F$_1$	BM + LG	2	2/16	—	—	—	
41	(A × C57BL)F$_1$	A immune 138/2	BM	5	1/27, 1/30, 3/41	31	138/2(C57BL)	0/3	Local mass and metastases.
39	(A × C57BL)F$_1$	A immune 138/2	BM + LG	5	1/17, 1/38, 1/41, 1/63, 1/121	52	138/2(C57BL)	1/2	
40	(A × C57BL)F$_1$	C57BL, immune Sa.I	BM	4	3/42, 1/52	30	Sarcoma I (A)	3/4	Local mass and metastases.
38	(A × C57BL)F$_1$	C57BL, immune Sa.I	BM + LG	7	1/6, 1/7, 1/17, 1/20, 1/21, 1/22, 1/25	—	—	—	
63									
59	A	(A × C57BL)F$_1$	BM	3	1/45, 1/62, 1/78,	40	138/2(C57BL)	2/3	Local mass, spleen^{++}.
58	A	(A × C57BL)F$_1$	BM + LG	5	1/5, 1/9, 1/25, 1/47, 1/79	39	138/2(C57BL)	2/2	Local mass and metastases.
69	C57BL	(A × C57BL)F$_1$	BM	5	1/7, 1/4, 1/40, 2/42	24	Sarcoma I (A)	1/3	Local mass, spleen^{+} and L. gland^{+}.
60	C57BL		BM	4	1/63, 1/70, 1/116, 1/150	36	Sarcoma I (A)	3/4	
62	C57BL	(A × C57BL)F$_1$	BM + LG	8	5/7, 1/14, 1/21, 1/48	26	Sarcoma I (A)	1/1	Local mass.
61									

TABLE 6.—*Survival rates in reciprocal semi-isologous chimaeras (corrected data from table 5)*

Chimaeras	Donor tissue		Number of survivals
	BM	BM + LG	Total
(A × C57BL)F_1 ← A	0/5	2/5	2/10
(A × C57BL)F_1 ← C57BL	1/4	0/7	1/11
Total	1/9	2/12	3/21 (14%)
(A × C57BL)F_1 → A	2/3	2/5	4/8
(A × C57BL)F_1 → C57BL	5/9	1/8	6/17
Total	7/12	3/13	10/25 (40%)

immunity was conferred on the chimaera through the immunized donor tissue; *e.g.*, an (A × C57BL)F_1 host that received BM + LG from an A donor immunized against the C57BL strain lymphoma 138/2 succumbed to this tumor; and when the 4 F_1 hosts were given BM from a C57BL donor immunized against Sarcoma I, the chimaeras all took, and died from Sarcoma I.

On the other hand, when the donor was (A × C57BL)F_1, it conferred susceptibility to the host for tumors of the other parental strain. The data show that the chimaeras became "neutral" to the tumor of the strain, which was represented only in the donor; *e.g.*, a C57BL host, given (A × C57BL)F_1 donor tissue, became susceptible to sarcoma I, and an A host to 138/2 lymphoma of C57BL. Trentin (*14*) and Main and Prehn (*15*) obtained similar results by using skin transplantation to test the immune response in semi-isologous chimaeras. In view of the fact that irradiation alone can produce an immunologically nonspecific neutral state, further experiments are in progress to ascertain whether or not the susceptibility of the parental strains was conferred on the chimaeras by the donor tissue.

Chimaeras with Hybrid Constitution

Table 7 shows a variety of chimaeras, that are composed of more than 2 strains. The number of survivors appears to be very small indeed; out of the 90 chimaeras only 11 lived more than 55 days. When, however, a correction is made for those chimaeras that died from tumor before 55 days without showing the serious signs of secondary disease, the number of survivors was found to be 24.

The data of table 7 also suggest that the addition of lymph gland to marrow as donor material reduced the survival rate; while 41 percent of "BM" chimaeras survived the secondary disease, only 20 percent of "BM + LG" chimaeras succeeded in so doing. It can also be seen that in

the (A × C57BL)F_1 hosts which were given either CBA or C3H donor material immune to Sarcoma I the immunity of the donor was not transferred to host. The chimaeras, when challenged with Sarcoma I as early as 26 and 30 days after irradiation, accepted and died of the tumor. The behavior of chimaeras with hybrid constitution corroborates the inferences based on the study of homologous and semi-isologous chimaeras.

Multiple Tumor Challenge

Several isologous, syngenetic, and homologous chimaeras were challenged with tumors successively. In some cases the implanted tumors were of the same strain origin, *e.g.*, Sarcoma I and MT03 both of strain A, but usually they were of different strains. The intervals between the tumor challenges varied from 3 to 8 weeks. The 1×10^6 tumor cells were injected alternately into the flanks of mice; if only 2 tumors were used, each flank received 1 tumor graft; if 3 were implanted, 1 flank received 2 tumors, the interval between the first and third challenge being not less than 12 weeks. The response of chimaeras to multiple tumor challenge is shown in table 8. The selected data represent the results of 6 out of the 11 experiments in which the chimaeras were challenged with more than 1 tumor.

In experiment 14, four CBA/CBA isologous "BM" chimaeras were given Sarcoma I, 46 days after irradiation. Four weeks later there was no sign of Sarcoma I, and the chimaeras were assumed to be immune to this tumor. However, one of the chimaeras began to develop a local growth noticed 49 days after the grafting of Sarcoma I, which increased in size very rapidly, and the sick mouse had to be sacrificed 16 days later because of a large ulcerating tumor. The other 3 individuals were free of Sarcoma I and were challenged with BP8. This tumor originated in C3H but it also takes well and grows in mice of CBA strain. It was, therefore, not surprising that all 3 chimaeras challenged died from the tumor. It grew, however, very slowly and killed the mice only 42, 44, and 51 days after transplantation. The position and structure of the last tumor was peculiar and a transplantation test was carried out to identify the nature of the local mass. Pieces of tumor were grafted by trocar into C3H and A mice and they grew in both strains.

The influence of the second graft on the first tumor, which may be considered to be an "enhancing effect," is suggested by the behavior of CBA/CBA chimaeras that had been challenged three times (expt. 2). The first tumor was Sarcoma I, which failed to grow; second was MT13, a C3H tumor which remained "latent" for 57 days but began to grow rapidly in 3 chimaeras after the third tumor challenge. The 3 individuals had to be sacrificed because of the MT13 tumor, 18 days after challenge with the third tumor (BP8). The latter grew only in one, killing the chimaera 62 days after it had received BP8 tumor by subcutaneous injection.

Experiment 7 shows the behavior of CBA/C3H chimaeras that were challenged with 4 tumors. It is interesting to note the degree of immu-

TABLE 7.—*Survival rate and immune response of chimaeras with hybrid constitution*

Expt. No.	Host	Donor	Donor tissue	Number of chimaeras	Number dead / Day of death	Age of chimaera at tumor challenge (*in days*)	Tumor	Tumor positive / Number challenged
42	(A × C57BL)F_1	CBA immune Sa. I	BM	5	1/24, 2/25, 1/47, 1/51	30	Sarcoma I (A)	1/2
66	(A × C57BL)F_1	CBA immune Sa. I	BM	3	3/30	—	—	—
43	(A × C57BL)F_1	CBA immune Sa. I	BM	3	1/40, 1/42, 1/87	32	Sarcoma I (A)	3/3
47, 70	(A × C57BL)F_1	CBA immune Sa. I	BM + LG	10	2/7, 3/8, 1/9, 1/20, 1/22, 1/31, 1/47	26	Sarcoma I (A)	1/2
67	CBA	(A × C57BL)F_1	BM	5	1/34, 3/39, 1/40	23	Sarcoma I (A)	1/5
48	CBA	(A × C57BL)F_1	BM + LG	5	1/4, 1/20, 1/21, 1/23, 1/24	—	Sarcoma I (A)	—
64	(A × C57BL)F_1	C3H immune Sa. I	BM	5	1/34, 3/42, 1/52	26	Sarcoma I (A)	5/5
46, 62	(A × C57BL)F_1	C3H immune Sa. I	BM + LG	10	1/6, 4/13, 1/44, 1/46, 1/47, 1/50, 1/53	26	Sarcoma I (A)	5/5
68	C3H	(A × C57BL)F_1	BM	5	1/13, 1/14, 1/17, 1/24, 1/47	—	—	—
44a, 44b	(A × CBA)F_1	C3H immune Sa. I	BM + LG	8	1/11, 1/24, 1/28, 1/29, 1/30, 1/36, 1/40, 1/41	—	—	—
19	CBA	R	BM	5	1/32	24	Sarcoma I (A)	0/5*
20	CBA	R immune Sa. I	BM + LG	10	1/31, 1/35, 2/54	24	Sarcoma I (A)	0/10
49, 50, 51	C3H—immune Sa. I	R	BM + LG	16	1/4, 1/7, 1/8, 3/9, 3/10, 2/12, 2/13, 2/14, 1/17	—	—	—

* Four were challenged 32 days later with BP8—*see* table 8, expt. 19.

Vol. 20, No. 6, June 1958
463225—58——5

TABLE 8.—*Multiple tumor challenge of chimaeras*

Expt. No.	Host	Donor	Donor tissue	1st Challenge			2nd Challenge			3rd Challenge			4th Challenge		
				Tumor (strain)	Age of chimaera (*in days*)	Positive / Total	Tumor	Age of chimaera (*in days*)	Positive / Total	Tumor	Age of chimaera (*in days*)	Positive / Total	Tumor	Age of chimaera (*in days*)	Positive / Total
14	CBA	CBA	BM	Sarcoma I (A)	46	1/4	BP8 (C3H)	106	3/3	—	—	—	—	—	—
2	CBA	CBA—immune Sa. I	BM + LG	Sarcoma I (A)	22	0/5	MT13 (C3H)	67	3/5	BP8 (C3H)	124	1/2	—	—	—
7	CBA	C3H	Spleen	MT03 (A)	102	1/4	Sarcoma I (A)	126	0/3	138/2 (C57BL)	166	0/3	BP8 (C3H)	196	1/3
19	CBA	R	BM	Sarcoma I (A)	24	1/5	BP8 (C3H)	56	2/4	—	—	—	—	—	—
4	R	R	BM + LG	Sarcoma I (A)	25	0/5	151/1 (CBA)	48	2/4	BP8 (C3H)	94	0/1	—	—	—
3	R	R—immune Sa. I	BM + LG	Sarcoma I (A)	25	1/4	151/1 (CBA)	78	2/4	BP8 (C3H)	120	0/2	—	—	—

nity of these chimaeras against Sarcoma I and BP8. It is not improbable that the interaction of immune responses can either enhance or depress the growth of tumor grafts. In experiment 7 perhaps there is an instance of the latter phenomenon. The multiple challenge of CBA/R and R/R chimaeras with tumors (expts. 19, 4, and 3) provides other examples to show the variable immune responses which may operate in the same individual or in different chimaeras within the same group. It is interesting to note that mice of R stock have a C3H component, yet they show a high degree of immunity against the C3H tumor BP8. In experiment 3, the protective tissue was BM + LG from R donor immunized against Sarcoma I and only 1 chimaera was found to be nonresistant to this tumor, which had a latent period of 140 days; during this time the same chimaeras were challenged with 2 other tumors—151/1 (CBA) and BP8 (C3H).

It is not improbable that the variable response of chimaeras to tumor challenge may be due to the fact that the ratio of host-donor cell types differs in chimaeras. Thus tumor challenge may be a method other than skin grafting by which differences in this respect can be disclosed in chimaeras.

Chimaeras Induced by Subacute Radiation

Tumor transplantation experiments were carried out on chimaeras that had been induced by subacute irradiation. The dose of 1000 to 1500 r was delivered at very low intensity during 25 hours (*16*). It has already been mentioned that the period of "inactivation" of the immune mechanism is longer when the dose is higher; therefore the subacute radiation-induced chimaeras represented very suitable material to test immunological responses by tumor grafting.

The behavior of these chimaeras to tumor challenge is shown in table 9. The challenge was made when the chimaeras were 3½ to 4 months old. The data show the chimaeras are not neutral immunologically at that time, and the C3H donor has not conferred immunity against the CBA 151/1 lymphoma.

Histology of Chimaeras

The data presented indicate that the genetic relationship between host and donor is the most important factor on which recovery from radiation injury depends. When both host and donor are genetically identical, bone-marrow therapy can result in 100 percent survival after the host has been exposed to LD100 total-body X irradiation.

Under these conditions, death may occur, though very rarely among isologous chimaeras within 6 to 14 days after irradiation. In most of such cases, early death can be attributed to acute damage in the gastrointestinal tract. It is common experience that donor marrow does not protect from "intestinal death." Early death is more frequent among homologous than isologous chimaeras because in the former not only is there intestinal damage but also the complete restoration of hematopoiesis in the blood-forming organs can fail. It is very likely that the possible

TABLE 9.—*Subacute radiation-induced chimaeras*

Host	Radiation dose (*r/25 hr.*)	Donor	Donor tissue	First tumor	Age of chimaera (*in days*)	Tumor positive / Total	Second tumor	Age of chimaera (*in days*)	Tumor positive / Total
CBA	1000	CBA	BM	138/1(C57BL)	118	0/5	BP8(C3H)	191	5/5
	1200	CBA	BM	138/1(C57BL)	118	0/5	BP8(C3H)	191	5/5
	1500	CBA	BM	138/1(C57BL)	118	0/5	BP8(C3H)	191	5/5
CBA	1000	C3H	BM	151/1(CBA)	110	5/5			
	1200	C3H	BM	151/1(CBA)	110	5/5			
	1500	C3H	BM	151/1(CBA)	110	4/4			
CBA	1000	T6+	BM	Sa. I (A)	115	0/1	138/2(C57BL)	132	0/1
	1200	T6+	BM	Sa. I (A)	115	0/2	138/2(C57BL)	132	0/2
	1500	T6+	BM	Sa. I (A)	115	0/2	138/2(C57BL)	132	0/2

cause of the latter event lies in the genetic incompatibility of host and donor tissues. The histological evidence supporting this assumption will be described later.

The highest proportion of homologous chimaeras (including semi-isologous and syngenetic host-donor combinations) die from a *chronic illness*, referred to as "secondary disease" by Congdon and Urso (*13*). These animals may fail to gain weight after the characteristic weight loss which accompanies total-body X irradiation within 5 or 6 days. Their movement remains sluggish, fur becomes ruffled, eyelids are closed, and the animals may show signs of dermatitis on the snout, ears, and tail. The symptoms of secondary disease in our chimaeras include severe diarrhea, which may account for the drastic weight loss (*17*); thus some of the emaciated, hunched chimaeras weigh only 8 gm. at death, losing 18 to 20 gm. during 4 to 5 weeks. Chimaeras suffering from "secondary disease" seldom show graying of hair. The lack of activity in the hair follicles is an indication that the general growth potentialities of the animal have been impaired. The death of chimaeras occurs at varying times; in our experiments they usually died between 25 and 50 days after radiation.

Several chimaeras in a moribund state because of the "secondary disease" were sacrificed and their various tissues studied. It was a most notable finding that the sternal and femoral *marrow* was filled with cells in various stages of hematopoiesis. Small, but very active, erythropoietic and myelopoietic centers that were sited around the portal vein (figs. 1 and 2) were also seen in the liver of some homologous chimaeras. In the *spleen*, the primary nodules and germinal centers of the white pulp were reduced in number and size. The spleen of chimaeras suffering from "secondary disease" was smaller than usual and mostly composed of red pulp and extensive fibrosis.

Liver lesions were rather common in certain isologous and homologous chimaeras. Histologically the lesions were of two types although their gross morphology was the same. One type appears 7 to 10 days after irradiation as superficial miliary foci of necrosis (fig. 3). Similar lesions were seen in 42- to 47-day-old chimaeras having "secondary disease" (fig. 4). The damage seems to be due to a functional disorder; the liver capillaries are congested and the obstruction causes anoxic conditions which result in parenchymal necrosis. While in the latter type of liver necrosis, dividing cells and large numbers of leukocytes are present indicating a certain degree of repair, no such signs were observed in the lesions of early chimaeras. The early liver lesions in our chimaeras also resemble that of Tyzzer's disease, caused by *Bacillus piloformis*. It is possible that the irradiation, by suppressing the defense mechanism of the host, enabled a chronic, but hitherto undetected, bacterial infection already present to increase and cause serious tissue damage, but no bacteria were identified in the sections. It is interesting to note that similar lesions of widespread focal, acute, nonspecific necrosis have been observed in livers of rats in which the defense mechanism has been blocked by zymosan (*18*).

The *gastrointestinal* tract did not always show serious damage in spite of the severe diarrhea present. Very often only slight hemorrhagic lesions could be seen in the gut. Frequently, the capillaries of the whole intestinal tract and those of the mesentery were dilated and engorged with blood. Dividing cells in the crypts of Lieberkühn were numerous, indicating that the replacement of goblet cells was in progress. Cell debris and degenerating cells with deeply stained pyknotic nuclei were always present, probably due to the irradiation which irreparably damaged the nuclear mechanism of these cells. In dying chimaeras, intussusception in various regions of the gut was also seen. Drastic injuries were observed in the intestinal tract of a few chimaeras moribund from secondary disease (figs. 5 and 6). The damage was most severe in the small intestine, affecting the villi. Sloughing of the mucous membrane and hemorrhage in the edematous villi were the most common features of the intestinal lesion.

Small intra-alveolar hemorrhage was found in the *lung* of a few chimaeras and, in one, the *kidney* contained a large abscess.

The *testes* of both isologous and homologous chimaeras were atrophic and greatly reduced in size; the germinal epithelium of the seminiferous tubules had disappeared and the lumen was filled with a mucous substance in which a few scattered degenerated cells could be seen (fig. 7). The interstitial connective tissue was scanty and in some regions represented only by very few cells. Hemorrhage in seminiferous tubules was also encountered in some chimaeras. The recovery of germinal epithelium was very variable; on the one hand, 150-day-old isologous chimaeras were seen to have entirely atrophied tubules; on the other, some semi-isologous chimaeras were found in which activity had returned in a few seminiferous tubules 56 days after irradiation (fig. 8). The miotic division of primary spermatocytes, however, was abortive, and the cells failed to mature into spermatozoa.

Because lymph glands are important in determining the character of immune responses, they were made the subject of careful analysis and their response was compared in the various chimaeras. Lymphoid organs are extremely sensitive to irradiation; a few hours after an LD50 total-body X irradiation the cells of lymph glands disintegrate and within 48 hours the architecture is disorganized. The lymphoid components disappear leaving the reticuloendothelial cells as the most obvious cellular elements in the nodes. The recovery of glands from the effect of radiation is a rapid process; it begins 4 or 5 days after irradiation, and 12 to 14 days later the primary and secondary centers are reformed (*19*). When mice are exposed to an LD100 total-body X irradiation, the destruction of the lymphoid cellular components in the nodes is accomplished within 3 or 4 days, but there is no sign of recovery up to the time of death (figs. 9 and 10). Recovery does occur only if the mice exposed to the LD100 were given marrow tissue from a nonirradiated donor. Cytological evidence has been provided that recovery is due to repopulation of the hematopoietic sites by donor cells (*3*, *20*).

One of our aims was to transfer lymph nodes of nonirradiated donors to irradiated hosts in order to influence the immunological behavior of the recipients. In view of the findings of Mitchison (*8*), a difference in behavior between "BM" and "BM + LG" chimaeras could be expected. When homologous chimaeras were given BM and LG donor material, however, the first very disturbing effect was the great increase in the death rate. Investigation was made, therefore, for the cause that could be responsible for the very low survival rate of such chimaeras.

As a preliminary investigation, a comparative histological analysis was made of the various tissues of isologous chimaeras (CBA/CBA) which received BM or BM + LG donor material. This investigation showed that the recovery of lymph nodes and spleen, which are the chief sites of lymphopoiesis, was the same in both kinds of chimaeras. The normal architecture of these organs was re-established about the 15th day after irradiation (figs. 11–14). The rate and degree of repopulation of the hematopoietic tissues by donor cells appeared to be similar. These findings are in line with the observation that the survival rate of "BM" and "BM + LG" isologous chimaeras is the same.

When, however, LG material was included as donor tissue for semi-isologous, syngenetic, and homologous chimaeras, the death rate of animals was always higher and death occurred earlier in the "BM + LG" chimaeras than in those that received only BM. While most of the latter died between 25 and 50 days after irradiation from "secondary disease," casualties among the "BM + LG" chimaeras began 4 days after irradiation, particularly when the donor was immunized. The majority of such chimaeras die within 14 days. The early death of "BM + LG" chimaeras could not in every case be attributed to intestinal damage or hematopoietic injuries. The gut appeared to be unimpaired; the sternal and femoral marrow often showed complete, or at least partial, restoration of hematopoiesis. The uncommonly large lymph glands were found to be loaded with cells like large lymphocytes so numerous that the germinal centers were obliterated (figs. 15 and 16). In the spleen the red pulp was filled with lymphocytes. Such a cellular hyperactivity closely resembles that seen in the lymphoid organs of immunized mice (*21*). Because the lymphoid components of the host have been destroyed by the radiation, the increased cellularity must be attributed to the influx of donor cells, which had been directly transported to and localized at these sites.

Those chimaeras which die later between the 25th and 50th day after radiation from "secondary disease" presented a different picture. The lymph follicles of lymph nodes and spleen remain atrophic (figs. 17–20). No difference was found in this respect between the "BM" and "BM + LG" chimaeras. Even in those chimaeras that survived or escaped "secondary disease," the lymph glands and spleen showed incomplete restoration of cellularity.

It is interesting to note that Billingham and Brent (*22*) have observed in mice made immunologically tolerant by the injection at birth of homologous spleen cells a condition similar to secondary disease in radiation

chimaeras. This condition they referred to as "runt disease" and attributed it to an immunological reaction on the part of the injected spleen cells against the host.

It is now presumed that the "secondary disease" which usually terminates in the late death of the chimaera is due to genetic incompatibility between donor and host resulting in a chronic tissue antigen-antibody reaction. The histological peculiarities of the lymphopoietic organs of the "BM + LG" chimaeras which die early indicate that death may be brought about by a similar but *acute antigen-antibody reaction.*

Discussion

Nature of Tissue Incompatibility in Chimaeras

It has already been mentioned that the survival rate of chimaeras is affected by the genetic constitution of host and donor, and the failure of the injected marrow to protect mice exposed to LD100 total-body X irradiation is attributed to incompatibility between the tissues of host and donor. Tissue incompatibility in mice is determined by genes of varying potency, the most important and "strongest" of which is the gene complex of the *H-2* locus, at which 10 alleles have already been detected and analyzed (*23*). The strains used in our experiments have the following *H-2* allele constitution and phenotype.

Strains	*H-2* alleles	Phenotype*
CBA	$H\text{-}2^k$	CdEfK
C3H	$H\text{-}2^k$	CdEfK
A	$H\text{-}2^a$	CDEFK
C57BL	$H\text{-}2^b$	D^bEFK^b
101	$H\text{-}2^k$	CD^kEfK
T6+ PCT	HYBRIDS	

*Analyzed by Dr. P. Gorer (personal communication).

It can be seen that the host and donor combination in our semi-isologous, syngenetic, and homologous chimaeras involves genotypes differing in the *H-2* locus. Those chimaeras, in which host and donor are identical, *i.e.*, the isologous chimaeras, have the highest survival rate, those where host and donor differ markedly have the lowest survival rate.

The importance of genetic differences at the *H-2* locus in determining mortality rates has been clearly shown by the experiments of Uphoff and Law (*24*). They employed mice of 2 coisogenic lines of strain C57BL/10 as host and donor. The 2 lines are genetically identical (isogenic) except for the *H-2* locus; one line being $H\text{-}2^b$, the other $H\text{-}2^d$, the latter having originated by mutation (*25*). The 60-day mortality in the "coisologous" chimaeras varied between 40 and 100 percent, depending on the genetic constitution of the donor.

In our CBA/C3H chimaeras the survival rate is only 70 to 75 percent in spite of the fact that CBA and C3H strains share the same allele *H-2*^{k}. Our observation suggests that besides the *H-2* locus other genes, *e.g.*, *H-1* and *H-3*, may also play a role in this histocompatibility reaction (*6*, *7*).

Accepting the evidence that the high mortality among homologous chimaeras is due to genetic incompatibility, the question is, how is tissue incompatibility expressed between host and donor? Is the secondary disease due to the reaction evoked by the host against the donor tissue or to that of the graft against the host?

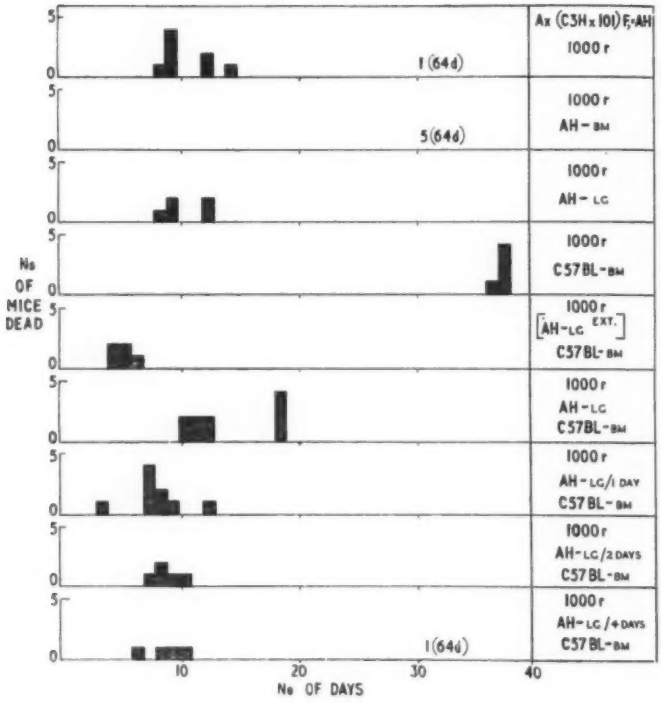
It is a known fact that animals react against tissues which are derived from donors of different genotype. This ability of the living organism is the basis of graft rejection. In order to act, however, the host's mechanism of immunity must recognize the antigens as foreign; this is then followed by a state of sensitization in which process the formation of antibodies is of great importance. It is possible to interfere with both processes; the recognition of certain foreign antigens by the lymphoid elements of adult mice can be specifically obviated by pretreating the animal in embryonic life with the same foreign antigens; or the formation of antibodies can be prevented by irradiation.

When the radiation dose is sublethal, bone-marrow therapy is ineffective in mouse chimaeras in which host and donor are genetically different. The failure of marrow to protect against radiation death is due to incomplete inactivation of the host mechanism of immunity; the donated bone marrow is rejected (*26*, *27*).

The efficiency of the host's reaction is clearly demonstrated when lymph-gland material from nonirradiated mice of host type was injected or a similar effect was observed when a host gland was protected during irradiation. Text-figure 2 shows a series of histograms, which illustrate the mortality among these chimaeras in which the host AH, a hybrid of $A \times (C3H \times 101)F_1$ cross, received nonirradiated lymph glands or had a gland protected. It can be seen that, when one gland of the host has been exteriorized and shielded by lead during the delivery of 1000 r, mortality was 100 percent in spite of homologous bone-marrow therapy. Because the cells of the nonirradiated lymph gland were capable of being activated by the antigens of the donor tissue and could form antibodies against the donor marrow, it is reasonable to assume that the graft was destroyed by the immune reaction. The time of death of such chimaeras suggests that restoration of hematopoiesis did not take place in the host.

In view of these facts it is conceivable, therefore, that in chimaeras which are affected by secondary disease a similar process is taking place, *i.e.*, the immune mechanism of the host is being restored, and the host is reacting gradually against the donor marrow cells. However, there are several other facts which do not support such an explanation.

1) Histological studies of the lymphoid organs in homologous chimaeras suffering from secondary disease have shown that the architecture and cellular organization of lymph glands and spleen are abnormal, and very often atrophic. Their appearance is very different from that shown by



TEXT-FIGURE 2.—Histograms showing the influence of lymph-gland donor material on the survival of chimaeras. The host is A × (C3H × 101) or AH. Data in column on *right* indicate the various treatments of the host:

1) Control, 1000 r without bone-marrow therapy.
2) 1000 r + isologous BM.
3) 1000 r and intravenous injection of cell suspension from isologous lymph gland (LG).
4) 1000 r and C57BL homologous BM.
5) One LG of host was exteriorized and lead-shielded, followed by 1000 r and bone-marrow therapy from C57BL.
6) 1000 r followed by bone-marrow therapy from C57BL donor, to which was added cell suspension of LG of AH.
7–9) Similar treatment, but LG was added 1, 2, or 4 days after homologous bone-marrow therapy.

Each square = 1 dead mouse.
5(64d) = 5 mice still alive at 64 days.

lymphoid organs during the process of immunization of the host. Our histological findings suggest that the sites concerned with the mechanism of immunity of the host are so grossly impaired as to be incapable of normal activity.

2) Our investigation brought to light that the F_1 hybrid is better as donor than host; the 55-day survival rate is 40 percent when P_1 or P_2 (parental strains) is the host and F_1 hybrid is the donor, while it is only

14 percent when F_1 is the host and P_1 or P_2 is the donor (table 5). The explanation of this difference is clear; the donor tissue of F_1 hybrid does not react against the host tissues of parental strains because it contains all the antigenic components of P_1 and P_2. In our case, (A $\times$ C57BL)F_1 has CDEFK/D^bEFK^b antigens, CDEFK from the A and D^bEFK^b from the C57BL parent strain. On the other hand, the P_1 or P_2 donor tissue can be immunized against the antigens of the hybrid set, since the hybrid contains antigenic components from the other parental strain, which may be different from those antigens that are possessed by the strain employed as donor. Thus, only the D^bEFK^b antigens are common in chimaeras of (A $\times$ C57BL)F_1 (host), C57BL (donor) combination; consequently, the CD-K antigens of the hybrid host will be able to stimulate the lymphoid elements of C57BL donor marrow to produce antibodies against the A antigens of the F_1 host.

The comparison of mortality rates in reciprocal semi-isologous chimaeras provides strong evidence that secondary disease is not a host reaction but, on the contrary, it is due to the reaction of donor tissue against the host's antigens. This possibility has already been suggested by Dempster (*28*) and Simonsen (*29, 30*). Formal experimental evidence that supports this assumption has been presented by Billingham *et al.* (*31*). Trentin (*32*) advanced some circumstantial evidence to suggest that a *graft* versus *host* reaction is involved in late radiation death of mouse chimaeras.

3) The ability of F_1-donor-marrow tissue to afford better protection than marrow from either parental strains has been demonstrated by Uphoff (*12*) and Uphoff and Law (*24*) with an interstrain combination like ours and several other of known genotype for *H-2* loci. The extensive data enabled them to establish the "F_1 hybrid effect" in bone-marrow therapy of homologous chimaeras and led to the conclusion that secondary disease and delayed death were due to immune response of the donor graft against the host.

4) The high incidence of *early* death of chimaeras that received BM + LG material is further evidence in favor of the graft reaction against the host. The LD100 radiation dose abrogates the ability of lymphoid organs in the host to react against foreign antigens and results in atrophied lymph glands, yet in homologous "BM + LG" chimaeras the histological studies have shown cellular hyperactivity in spleen and lymph glands indicating that an "immunizing process" is in progress in which only the transplanted donor cells could participate. Similar conclusions have been drawn by Simonsen (*29, 30*) in his investigation into the effect of adult homologous spleen cells on chick embryos.

5) If secondary disease is a manifestation of graft reaction against the host, then by using as donor material embryonic tissue which is immature and incapable of reacting immunologically, *i.e.*, it is still in the "null-period," the mortality rate in chimaeras in which host and donor are genetically different could be expected to be low. In a few recent experiments in this laboratory, tissue of 14- to 21-day-old C57BL embryos was used as donor tissue into CBA hosts. This combination, when using

bone marrow from adults, has been invariably lethal; the chimaeras die from secondary disease. In one experiment, 10 CBA were given adult bone marrow of C57BL and all died before 70 days; whereas 7 out of 10 CBA survived beyond this period when they were given embryonic material (unpublished observations). Diarrhea, when it appeared in the latter chimaeras, was mild, and the mice recovered. The results, which are preliminary only, seem to show that "antigenetically immature" tissue in certain host-donor combinations is more favorable as donor material than bone marrow of adult mice. Uphoff (*33*) used embryonic liver and demonstrated conclusively the great advantage of fetal donors in certain combinations.

6) The so-called "runt disease" of tolerant mice is very similar to "secondary disease" of radiation chimaeras. Billingham (*34*) brought forward evidence to show that runt disease is due to a "*graft*-versus-*host*" reaction.

Tissue Tolerance in Chimaeras

A number of homologous chimaeras living for many months have either overcome or escaped secondary disease. One may assume, as the most simple explanation, that in such chimaeras the donor tissue has been totally replaced by the host before it could set into motion an antigen-antibody reaction. There is ample evidence, however, to show that donor cells persist for many weeks and months in chimaeras and, in some, constitute the only active cells in hematopoietic organs (*20*).

In our CBA/T6+ chimaeras, the chromosomally marked T6 cells were identified by Dr. C. E. Ford, 315 days after the donor cells had been injected into the irradiated CBA host. In the homologous rat chimaeras, Lindsley and his colleagues (*2*), by using immunological markers, were able to demonstrate the donor erythrocytes in the rat 147 days after irradiation. Nowell *et al.* (*4*) made similar observations by employing the positive alkaline-phosphatase reaction of rat granulocytes.

In order that donor cells can exist in a genetically different host, the antigenically different tissues of host and donor must undergo a process of adaptation in such a manner that they become nonreactive to each other. There is much evidence that tissues can become mutually "nonreacting." Thus Owen (*35*) found antigenically different erythroid components in the blood of dizygotic twins in cattle, Dunsford and others (*36*) in man. Owen and Ripley (*37*) have induced similar chimaerism in rats. Recently two more "blood chimaeras" have been found and analyzed in human twins (*38*, *39*).

Coexistence of genetically different cells can be brought about experimentally by injecting spleen cells of adult mice into embryos or newborn individuals (*34*). It is known that embryos and newborn animals are not capable of producing a state of sensitization to foreign antigens, *i.e.*, they are immature immunologically. When the maturation of the cellular components, representing the mechanism of immunization, takes place in the presence of a foreign antigen, the tissues of host will acquire a tolerance to this particular antigen. The outcome of this adaptation is

twofold: (*a*) the host cannot be sensitized by the donor tissue and (*b*) it cannot activate the donor marrow cells to produce antibodies. The "acquired tolerance" to the specific antigen, which was injected into the embryo or newborn animal, can be disclosed in skin-grafting experiments carried out in adult individuals.

It is very likely that in our radiation chimaeras which survive, a similar type of adaptation has taken place between the genetically different host and donor tissues. The recovery of host tissue from the effect of radiation occurs in the presence of foreign antigen or antigens represented by the donor marrow. Cellular adaptation, the nature of which has yet to be analyzed, is a mutual process affecting both host and donor tissue and results in tolerance between the genetically different tissues. In chimaeras that show secondary disease, adaptation has failed or is incomplete and permits the development of the chronic antigen-antibody reaction.

In our semi-isologous chimaeras the mutual unreactivity of the tissues has been detected by challenging the animals with tumors. It was found that the presence of donor cells conferred susceptibility for tumors of the donor strain; thus in A/(A × C57BL)F_1 chimaeras, the strain A host has accepted and grown a C57BL lymphoma, and *vice versa* C57BL/(A × C57BL)F_1 chimaeras became susceptible to Sarcoma I, a strain A specific tumor. Similar tolerance to skin grafts was observed in homologous chimaeras by Main and Prehn (*15*) and Trentin (*14*).

In a small-scale experiment, bone marrow of homologous chimaeras, which survived secondary disease, has been used again as donor material in a similar host-donor combination as previously. It was thought that when marrow of chimaeras in which the host-tissues have already developed tolerance is used as donor material, it might reduce the incidence of death due to antigen-antibody reaction. In our particular experience, however, several of the new "secondary" chimaeras have succumbed to the chronic disease, which shows that the donor marrow obtained from a chimaera did not enhance the process of adaptation in the "secondary" chimaera. This in fact suggests that a second process of adaptation must occur in the new recipient in order that the host tissue and "chimaeric" donor marrow may become mutually nonreacting in this second symbiotic state.

Further investigation into the behavior of radiation chimaeras should reveal more about the nature and range of cellular adaptation of this kind. The variable response shown by chimaeras to multiple tumor challenge seems to indicate that a wide range of tolerance can be brought about between the tissues of host and donor.

Summary

Radiation chimaeras are mice that due to bone-marrow therapy survive LD100 total-body X irradiation. The donor material may differ genetically from the irradiated recipient. According to the genetic relationship between the host and donor, radiation mouse chimaeras can be grouped

as follows: 1) isologous, 2) semi-isologous, 3) syngenetic, 4) homologous, and 5) heterologous.

The immunological responses of the various chimaeras have been analyzed by challenging them with different tumors. In *isologous chimaeras*, in which host and donor are of the same genetic constitution, *i.e.*, are members of the same strain (CBA/CBA), the donor marrow does not fully restore the immune mechanism of the irradiated host. The addition of lymph gland to bone marrow as donor material shortened the immunologically neutral state of isologous chimaeras and almost completely restored immunity of the host to foreign tumor. In *syngenetic chimaeras*, which are composed of more than 2 strains, one or more of these being common to host and donor, the addition of lymph gland to bone marrow as donor material fully restored natural immunity of host. In *homologous chimaeras* in which host and donor belong to different strains, it was found that the animals were not neutral immunologically and that the immune mechanism was not controlled by the host alone. *Semi-isologous* chimaeras are mice of 1 of 2 inbred strains, *e.g.*, (A or C57BL) in combination with the F_1 hybrid. While immunity on the (A $\times$ C57BL)F_1 host was not conferred by the marrow and lymph-gland donor material of the parental strains that have been immunized against tumors of each other, bone marrow and lymph gland of the F_1 hybrid seemed to confer susceptibility on mice of the parental strains for tumors of each other. A similar immunological response was demonstrated by chimaeras with hybrid constitution and by chimaeras that received *subacute* radiation. Chimaeras, when challenged by different tumors in succession, exhibited variable immune responses that may suggest tumor "enhancement," interaction, or differences in the proportion of host-donor cell population.

Chimaeras in which host and donor differ genetically show varying degrees of "secondary disease," depending on the extent of genetic divergence. Secondary disease is attributed to a *chronic* tissue antigen-antibody reaction. The early death of chimaeras that received bone marrow and lymph gland as donor material is assumed on histological evidence to be due to a similar but *acute* tissue antigen-antibody reaction. Experimental evidence has been provided which seems to indicate that the immune reaction is that of the *graft* versus *host*. It is argued that in chimaeras which survived or escaped secondary disease, the donor and host cells have become "nonreacting" *i.e.*, have been altered immunologically and have been adapted to an existence in "the symbiotic state."

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PLATES

463225—58——6

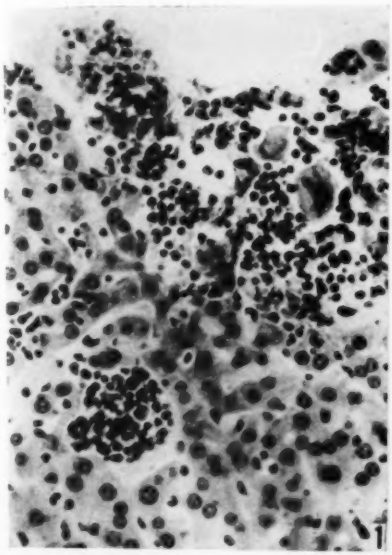
PLATE 106

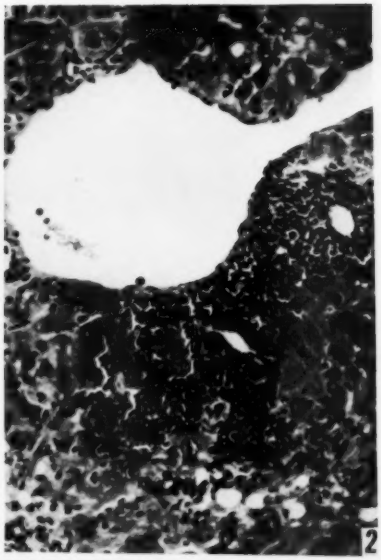
FIGURE 1.—Erythropoiesis in liver of a 42-day-old homologous chimaera (A × C57BL)F_1. × 200

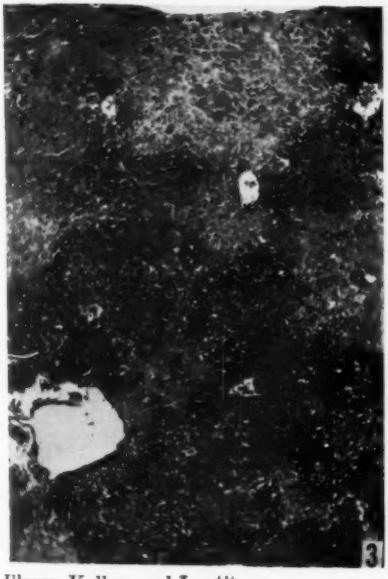
FIGURE 2.—Myelopoiesis in liver of a 41-day-old homologous chimaera (A × CBA)F_1. × 160

FIGURE 3.—Lesion in liver of a 7-day-old isologous chimaera (A × C57BL)F_1/(A × C57BL)F_1. × 45

FIGURE 4.—Lesion in liver of a 42-day-old semi-isologous chimaera (A × C57BL)F_1. × 60







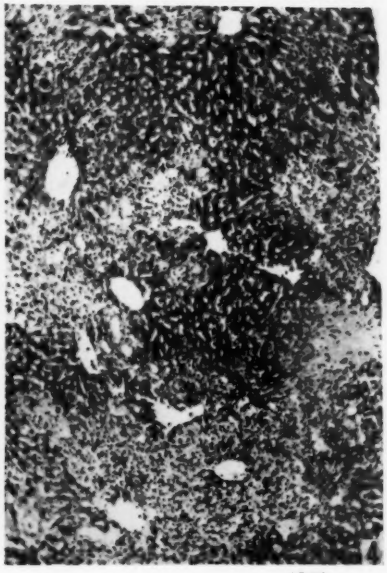


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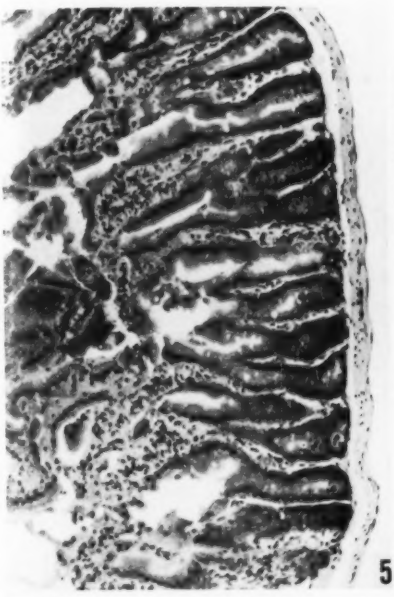
PLATE 107

FIGURE 5.—Damaged mucous membrane in intestine of a 21-day-old homologous chimaera; CBA/(A × C57BL)F_1. The villi are edematous and sloughing off the mucous membrane. Mitotic figures in the Lieberkühn crypts are abnormal. × 90

FIGURE 6.—Region of small intestine of a 42-day-old homologous chimaera; CBA/C3H, showing slightly damaged mucosa and a hyperactive lymph node. The donor material of C3H was BM + LG. × 40

FIGURE 7.—Section from testis of a 38-day-old homologous chimaera; CBA/R, showing inactive seminiferous tubules. × 120

FIGURE 8.—Section from testis of a 56-day-old homologous chimaera; CBA/R, showing partial recovery of spermatogenesis. No differentiation of spermatids into spermatozoa. × 90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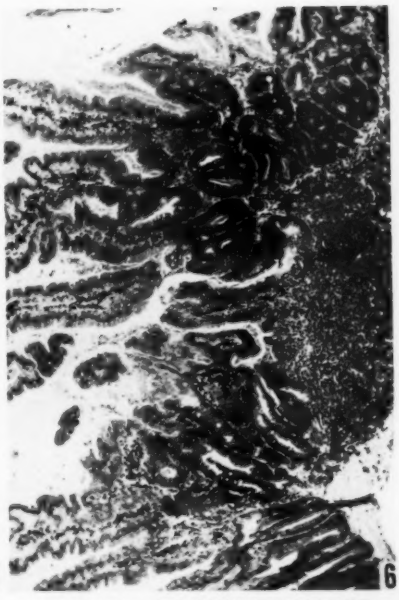
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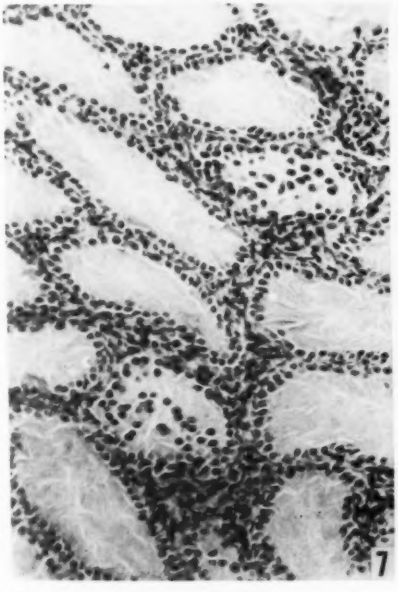
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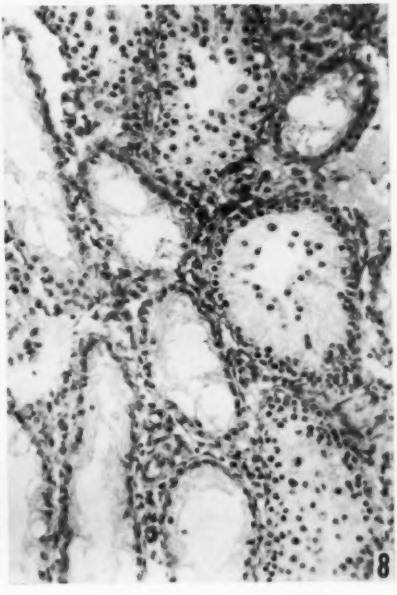
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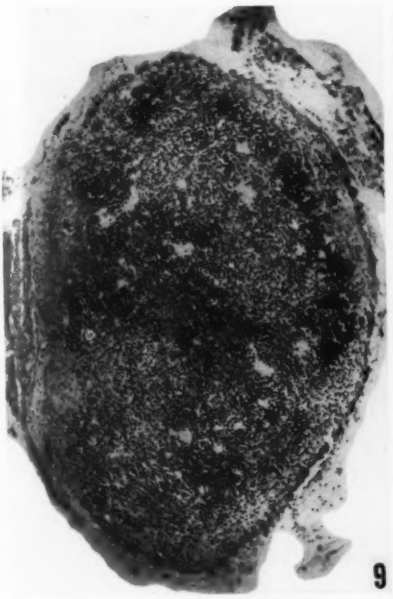
PLATE 108

FIGURE 9.—Inguinal lymph gland of a CBA mouse that received 950 r (LD100) total-body X irradiation—animal was sacrificed on the 8th day. Photomicrograph shows disintegration of germinal centers. × 40

FIGURE 10.—Same as figure 9, at higher magnification, to illustrate degenerating cellular components of lymph gland. × 250

FIGURE 11.—Axillary lymph gland in a 15-day-old isologous chimaera; CBA/CBA, which was given BM donor material. *Note* 4 peripheral regions which are being colonized by donor cells. × 40

FIGURE 12.—Same as figure 11, at higher magnification. × 250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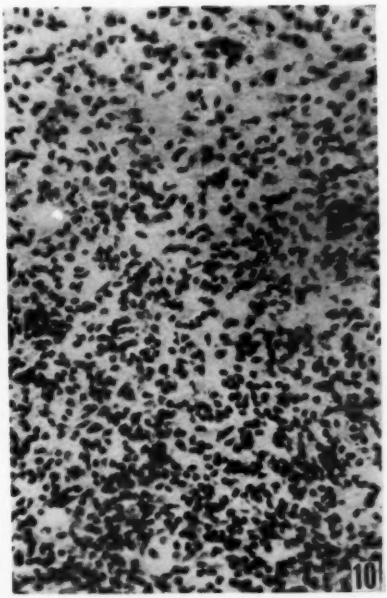
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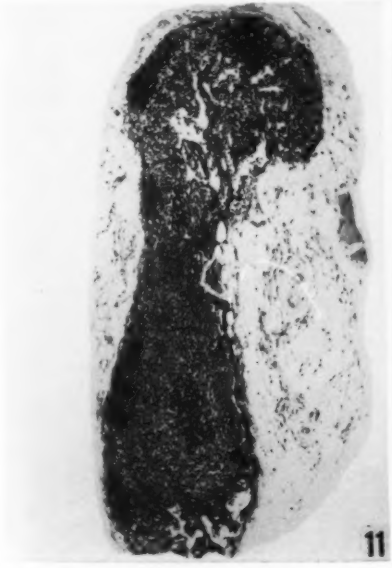
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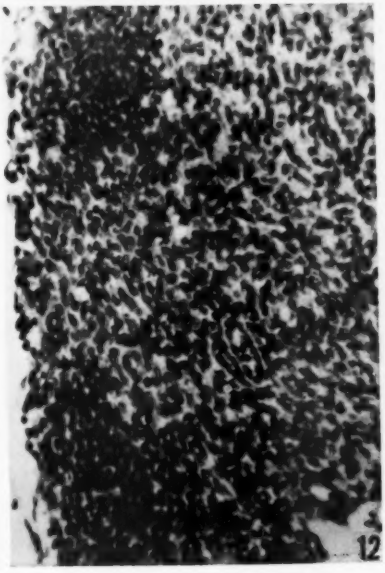
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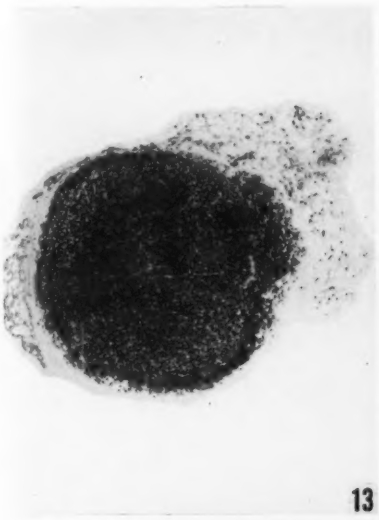
PLATE 109

FIGURE 13.—Axillary lymph node in a 15-day-old isologous chimaera; CBA/CBA, which was given BM + LG as donor material. Repopulation of lymph gland is more extensive than in gland shown in figure 11. × 30

FIGURE 14.—Same as figure 13, at higher magnification. × 200

FIGURE 15.—Lymph gland of an 8-day-old semi-isologous chimaera; (A × C57BL) F_1 host was given BM + LG as donor material from a C57BL mouse immunized against Sarcoma I. The cellularity of lymph node is increased (*cf.* fig. 9). × 30

FIGURE 16.—Same as figure 15, at higher magnification, to illustrate the cellular elements. × 200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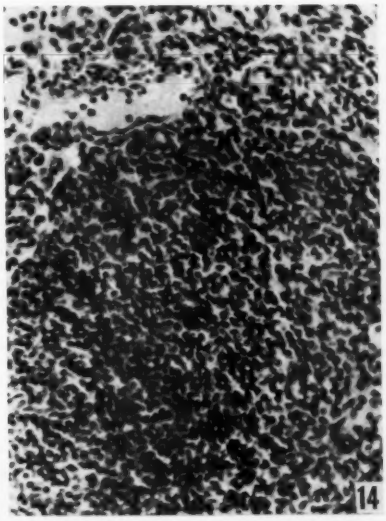
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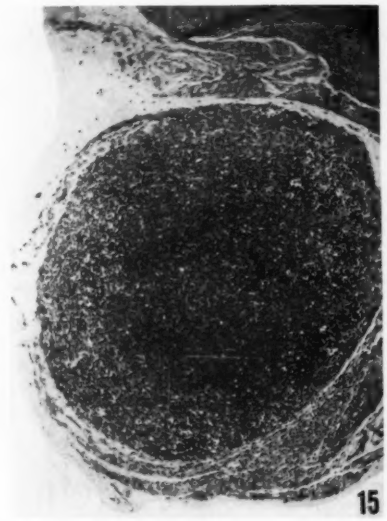
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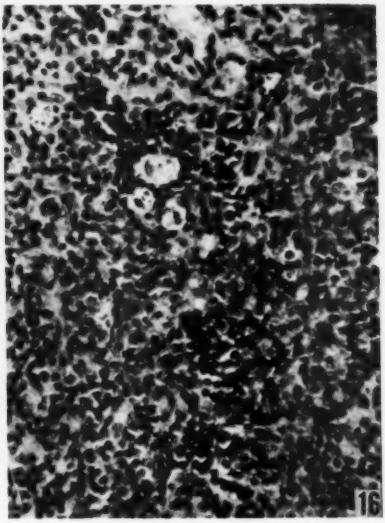
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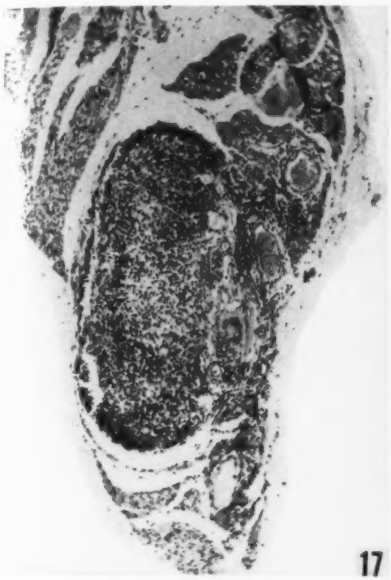
PLATE 110

FIGURE 17.—Inguinal lymph node of a 34-day-old homologous chimaera, CBA/R, the donor material was BM + LG. Photomicrograph shows the lack of histological organization. × 40

FIGURE 18.—Same gland, at higher magnification. × 250

FIGURE 19.—Spleen of a 31-day-old homologous chimaera; CBA/R, the donor material was BM + LG. Architecture of spleen is grossly altered, and remnants of white pulp can be seen around capillaries. Most of the red pulp is replaced by a fibrous collagen deposit. × 40

FIGURE 20.—Same as figure 19, at higher magnification, showing abnormal cellular elements of white pulp. × 250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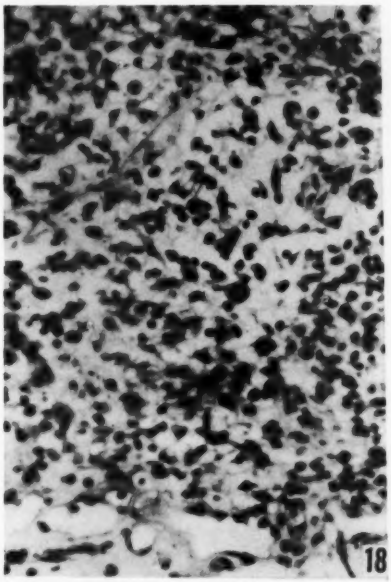
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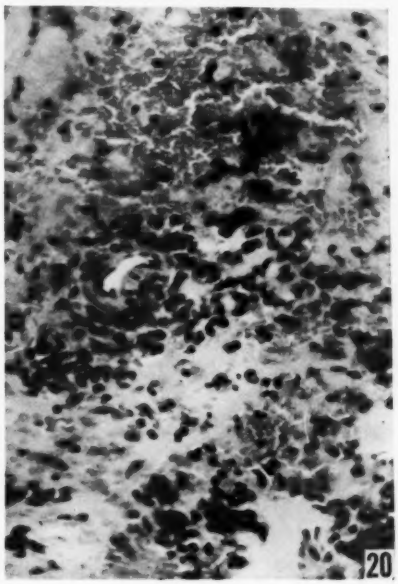
20

Response to Cortisone and Development of Cortisone Resistance in a Cortisone-Sensitive Lymphosarcoma of the Mouse [1]

JULIA MCCAIN LAMPKIN [2] *and* MICHAEL POTTER,[3]
Department of Physiology, The George Washington University School of Medicine, Washington, D. C., and National Cancer Institute, [4] *Bethesda, Maryland*

In 1944, Heilman and Kendall (*1*) reported regressions of established transplants of a mouse lymphosarcoma following treatment with compound E (cortisone). This discovery, along with the elucidation of the physiological effects of adrenocortical substances on normal lymphoid tissue (*2–6*), led to the use of cortisone in the treatment of human acute leukemia. Cortisone was found to produce striking but short-lived remissions in certain cases of acute leukemia in man (*7, 8*). Subsequent work with cortisone treatment of many of the commonly used transplantable mouse and rat lymphoid tumors failed to reproduce effects like those observed by Heilman and Kendall (*9–19*).

A possible explanation for these apparently irregular results to demonstrate regressions of lymphoid tumors might lie in the fact that many of the neoplasms had been transplanted for many years, whereas most of the Heilman and Kendall observations were made during early transfer generations. The differences observed might also be explained on the basis of certain differences in the nature of various lymphoid neoplasms of the mouse (*20–22*). At least three types of lymphoid neoplasms may be distinguished by their respective behavioral characteristics as subcutaneously transplanted tumors. The generalized lymphocytic neoplasm produces a moderate-sized local growth and intense dissemination to the viscera; this neoplasm grows very rapidly and kills its host at 7 to 20 days. In contrast, the "lymphocytic neoplasm—localized" (Dunn) or lymphosarcoma produces a massive local growth and, after attaining a large size, only rarely disseminates. A third type, a less mature lymphocytic neoplasm that resembles certain examples illustrated by Dunn [figs. 36 and 37 (*22*)], upon subcutaneous inoculation produced no local growth, or only a tiny local growth, but disseminates to the peripheral

[1] Received for publication November 21, 1957.

[2] Guest worker, Leukemia Studies Section, Laboratory of Biology, National Cancer Institute. Portions of this paper will be used by J. McC. L. in a doctoral dissertation being prepared under the sponsorship of the Department of Physiology, The George Washington University School of Medicine. This work was done under the support of a National Science Foundation predoctoral fellowship. *Present address:* National Cancer Institute, Bethesda 14, Md.

[3] The authors wish to express their thanks to Miss Beatrice C. Lampkin and Mr. Alvado F. Campbell for their technical assistance.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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and deep lymph nodes and viscera. The involvement of the spleen is particularly intense and this organ attains massive size. The growth process may require several months to kill the host animal. There are probably other types of lymphocytic neoplasms that are intermediate between the forms described above, as well as types that result from long-term laboratory transplantation, *i.e.*, loss of morphologic differentiation, or tumor progression. The Heilman and Kendall observations were made apparently on a lymphosarcomatous type of lymphoid neoplasm. In this laboratory many other lymphocytic neoplasms have been studied for their sensitivity to cortisone, but the lymphosarcomatous type of lymphocytic neoplasm appears to be the most sensitive.

The importance of studying lymphoid neoplasms in their early transfer generations is suggested by the observed influences on leukemogenesis in the mouse of adrenocortical substances. Kaplan *et al.* (*23*, *24*) have shown that when cortisone acetate and hydrocortisone were given, either immediately or 6 weeks following the last dose of X radiation, the onset of leukemia was delayed in C57BL mice. The cortisone treatment schedule employed by Kaplan was of 10 weeks' duration. Woolley *et al.* (*25*) demonstrated a similar inhibitory effect of cortisone on the development of spontaneous leukemia in the AKR mouse. Further, surgical removal of the adrenal glands of the mouse produces the opposite effect. Law (*26*) reported that adrenalectomy hastened the onset of spontaneous leukemia in C58 mice. Kaplan (*23*) later demonstrated a similar acceleratory effect of adrenalectomy on radiation-induced leukemia in the C57BL mouse.

In the present report, a lymphocytic neoplasm, P1798, of the BALB/c strain of mouse, was used as the object of study. This neoplasm is like the type employed by Heilman and Kendall in the following ways: it produced a massive subcutaneous growth, arose from a mediastinal structure, the thymus, and was studied during the early transfer generations.

Three effects of cortisone on neoplasm P1798 have been observed. They are: 1) the ability to induce regression of established tumors, 2) the ability to prolong the survival time of animals bearing subcutaneous or ascites transplants, and 3) the ability to evoke regressions of established ascites and inhibit the growth of ascites neoplastic cells during the period of logarithmic growth.

Materials and Methods

The neoplasm P1798 employed in this study arose in a strain BALB/c male mouse.[5] This mouse had received a 20 percent diethylstilbestrol-cholesterol pellet subcutaneously when 79 days old, and 165 days later had developed bilateral testicular neoplasms. The diethylstilbestrol pellet was removed, and regression of the testicular tumors followed (*27*). When the mouse was 463 days old, a diethylstilbestrol pellet was again

[5] Treated with diethylstilbestrol by Dr. H. B. Andervont.

placed subcutaneously. At 521 days of age the mouse showed severe respiratory difficulty and was sacrificed. At autopsy there was a large thymic mass filling the upper thorax; the liver, spleen, kidneys, and lymph nodes were normal.

Microscopically,[6] the tumors were composed of round cells with deeply basophilic nuclei and a narrow rim of cytoplasm. The nucleus usually contained a single small nucleolus. The cells were generally uniform in size and staining reaction. The normal architecture of the thymus was destroyed by infiltration with the neoplastic cells, which extended beyond the thymic capsule. Histologically, the tumor was characteristic of lymphocytic neoplasms in the mouse.

The thymic neoplasm was homogenized in a Potter-Elvehjem homogenizer and inoculated intraperitoneally into a group of mice for the initiation of the ascitic line, and subcutaneously for the initiation of the subcutaneous line. The tumor has been carried serially as an ascites tumor and also as a subcutaneous neoplasm in (BALB/c $\times$ DBA/2)F_1 hybrid mice. It has failed to grow in DBA/2Lw mice. Routine transfers of the ascitic line were accomplished by the inoculation of 0.1 ml. of ascites. The subcutaneous passages were accomplished by inoculation of tumor brei through a 21-gauge needle in the right flank. When dilutions of tumor material were made, the diluent was chilled, sterile Locke's solution.

The experimental animals were (BALB/c $\times$ DBA/2)F_1 hybrid mice supplied by Dr. George E. Jay, Laboratory Aids Division of the National Institutes of Health. BALB/c mice were obtained from the inbred colony of Dr. H. B. Andervont, and DBA/2Lw mice were obtained from the inbred colony of Dr. L. W. Law. They were housed in plastic cages, 6 to 8 mice per cage, and fed Derwood pellets and tap water *ad libitum*. At the time of inoculation of tumor, recipient mice were 1.5 to 3 months of age and weighed from 18 to 25 gm.

The growth or regression of subcutaneous tumors was determined by the techniques described by Schrek (*28*). The tumors were measured in three dimensions by a Lufkin Outside Caliper which had been filed down to make the edges parallel. The distance between the caliper arms was obtained by placing the edges on a millimeter rule. The length, width, and height away from the body were obtained, and the volumes were calculated according to Schrek's formula in which the tumor volume = $4/3\ \pi\ (\frac{d}{2})^3$ or $.5236\ d^3$. The change in volume was expressed as percent change of original volume for a given period.

The techniques for counting ascites tumor-cell populations were those described by Potter and Law (*29*). The peritoneal cavities of mice from which the skin had been removed were washed with three 25 ml. volumes of Locke's solution. Aliquots were counted in Levy-Hauser counting chambers with a binocular microscope employing 12.5$\times$ oculars and a 20$\times$ objective. Only cells that appeared to be viable and in good condition were counted. Histiocytes and red blood cells were excluded from the counts.

[6] Tissue sections of pathological material were kindly reviewed and described by Dr. Thelma B. Dunn.

The cortisone acetate was a commercial preparation obtained from Merck, and crystalline cortisone (Δ^4-pregnene-17α,21-diol-3,11,20-trione) and prednisone ($\Delta^{1,4}$pregnadiene-17α,21-diol-3,11,20-trione) were supplied by the Cancer Chemotherapy National Service Center. The crystalline compounds were prepared for injection by homogenizing the crystals in a clear solution of 0.5 percent carboxymethylcellulose (Ruger Chemical Company) in a Potter-Elvehjem tissue homogenizer. The carboxymethylcellulose was dissolved in .85 percent NaCl solution. The effects observed were the same with cortisone acetate or crystalline cortisone. No difference was found between controls given carboxymethylcellulose as a placebo or controls with no treatment. No preservative was used since preparations were always used within 1 to 2 days and were stored in the refrigerator at 4° C. during this time.

The drugs were administered subcutaneously in the interscapular region. Mice treated continuously with cortisone received 25 mg. per kg. for the first 7 days and 15 mg. per kg. subsequently. It was found that continuous treatment at the 25 mg. per kg. level produced severe weight loss and death. After the initial treatment with 25 mg. per kg. of 7 days, animals have tolerated 15 mg. per kg. for periods of 60 days or more without signs of severe toxicity.

Amethopterin (Methotrexate®)[7] was administered subcutaneously twice weekly at the dose level of 3 mg. per kg.

Results

Effect of Cortisone on Established Subcutaneous Transplants of Lymphosarcoma P1798

The observations on established subcutaneous transplants of P1798 are given in table 1. In these experiments the tumor was inoculated subcutaneously into the right flank and allowed to grow until it was approximately 2 to 3 cm.^{3} in volume when treatment was started. The results are recorded as the percent change from the original calculated volume. The weights of excised tumors at the end of 6 days are also recorded. More than 100 (BALB/c $\times$ DBA/2)F_1 mice in all the transfer generations studied to date showed virtually complete regression when cortisone was administered at 25 mg. per kg. daily for 5 consecutive days. By this time treatment had resulted in decrease of the tumor volume to negligible size (1% of original measurement), in contrast to the controls in which the tumor volume increased 142.4 percent during the same period of time. After 2 daily treatments with cortisone at 25 mg. per kg. the subcutaneous tumors on the average had decreased to 9.7 percent of the original volume.

When the animals were sacrificed on the 6th day, as they were in the specified transfer generations, the weight of the excised, cortisone-treated tumors varied from 20 to 100 mg. (average, 54 mg.). The average weight of the control tumors was 5.9 gm.

[7] Methotrexate was generously contributed by the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y.

TABLE 1.—*Effect of cortisone on established P1798 lymphosarcoma*: Effect of different dose levels of cortisone*

Transfer generation	Day begun†	Number of mice per group‡	Tumor volume on day begun ($cm.^3$)	Average change in tumor volume expressed as percent of original volume§								Weight (*in gm.*)	
				3 Days		6 Days						After 6 days	
				Cortisone dose (*mg./kg.*)		Cortisone dose (*mg./kg.*)						Cortisone dose (*mg./kg.*)	
				0	25	0	2.5	5	10	15	25	0	25
2	32	6–7	2.68	+67	−82	+105	+40.8	‖			−99.1	6.0	.098
3	19	6–8	1.17	+86.6	−93.6	+181	+45		−88.7	−96	−98	5.1	.100
4	18	8	1.43	+131	−97.3¶	+272		+17	−97	−97		6.7	.020
5	18	5	1.25	+138		+236	+61	+24	−46				
6	20	8	2.25	+77	−88.5	+195.5	+55.6	+.2	−33		−98		
7	17	8–32	2.27			+54.4	+46	+20.6	−29.6		−99.8		
8	20	8–24	4.34			+69.3		+38.8	−33		−99.7		
9	18	8	3.59			+107			−7.9	−65.8	−99.4		
7	18	14–16	1.23			+426					−98		

*All mice in each experiment were (BALB/c × DBA/2)F_1 hybrid, except in last experiment, generation 7, which were BALB/c.

†Day of original measurement after transplantation.

‡All mice were inoculated subcutaneously with 0.2 ml. of a homogenized subcutaneous growth.

§Value calculated from formula = $\frac{\text{volume of tumor after 6 days} - \text{volume tumor day treatment begun}}{\text{volume of tumor day treatment begun}}$.

‖=No observations were made at particular dose level.

¶Dose of cortisone was 15 mg./kg.

A gradual change in the response of the tumor to 10 and 15 mg. per kg. doses of cortisone was noted as the tumor was transplanted in succeeding generations. Through the fourth transfer generation a 10 mg. per kg. dose of cortisone had a profound effect in causing regression of the tumor, but by the ninth transfer generation the response had diminished to the extent that tumors did not significantly decrease in volume. It will be noted that cortisone, at levels below 10 mg. per kg., did not have an appreciable effect in causing regression of tumors. However, it is remarkable that, in the early transfer generations, doses as low as 2.5 mg. per kg. lowered the rate of growth of the tumor. There was no significant difference in response between males and females when tested in the same transfer generations. Figure 1 shows the striking regressions in an established neoplasm.

Established tumors in BALB/c mice treated with 25 mg. per kg. of cortisone decreased to an average of 2 percent of the original volume in 6 days. In contrast to (BALB/c × DBA/2)F_1 hybrids, the tumors in BALB/c control mice grew much faster, with an average increase of 426 percent in volume in 6 days.

Effect of Cortisone on Survival Time of Mice Bearing Subcutaneous Transplants of P1798

Two types of experiments were employed in studies of survival time. In the first, cortisone was begun 24 hours after the inoculation of the tumor-cell suspension. The second type of experiment involved the administration of cortisone to established subcutaneous transplants. In all cases the dose of cortisone was 25 mg. per kg. per day for the first 7 days and 15 mg. per kg. per day thereafter.

TABLE 2.—*Effect of cortisone on survival time of mice bearing subcutaneous transfers of P1798*

Transfer generation	Total No. daily treatments	Survival time (*in days*)						
		Control			Cortisone*			
		Number of mice	Number of mice surviving	Average day of death (*range*)	Number of mice	Number of mice surviving	Mice still surviving (*in days*)	Average day death
2	12,16,20	8	0	48. 1 (33–55)	24	23	306	†
10	61	15	0	31. 6 (27–45)	15	7‡	145	61. 7§
12	51	8	0	34. 2 (29–39)	8	8‖	116	¶

*Cortisone treatment started 24 hours after tumor inoculation. For the first 7 days the dose was 25 mg./kg. followed by a daily dosage regimen of 15 mg./kg.

†One mouse died of intercurrent infection at day 130.

‡All mice were alive without tumor at day 145.

§Average day of death of 8 mice that died of tumor.

‖All mice were alive without tumor at day 116.

¶No mice died in transfer generation 12 through the 116th day.

Table 2 records the effect of cortisone treatment begun 24 hours after inoculation of tumor. Cortisone treatment of mice bearing the second transfer generation of neoplasm P1798 resulted in complete suppression of tumor growth, though one group received cortisone for only 12 days. Studies with the tenth and twelfth transfer generations revealed that cortisone had retained its ability to suppress the growth of subcutaneously transplanted tumors. However, certain mice bearing tumors of the tenth transfer generation ultimately showed progressive growth of the tumor while on cortisone treatment.

In the second type of experiment, cortisone was given to mice with established tumors, as shown in table 3. Cortisone caused almost complete regression of these tumors by the 6th day of treatment. However, the tumors recurred despite continuous treatment and eventually killed the mice. It may be noted that even though there was ultimate redevelopment of tumor following regression, treated mice outlived the untreated controls. The differences are more pronounced if the survival time is based on the day cortisone was begun.

Development of Resistance to Cortisone

One of the purposes of this study was to determine whether a highly cortisone-sensitive tumor would develop resistance to cortisone. Heilman and Kendall described apparent resistance to cortisone in tumors that reappeared after cortisone had caused apparently complete regression. Drug resistance of lymphocytic neoplasms and other leukoses to certain antimetabolites has been described (*30–37*) and shown to be a permanent, heritable character of the population once the selective process has been completed (*38*). In view of the striking cortisone sensitivity, P1798 presented a useful model for a study of the development of resistance to this drug.

A drug-resistant subline was developed from the subcutaneously transferred line at transfer generation three by means of intermittent treatment of an established tumor. Beginning on the 18th day following the inoculation of tumor cells, cortisone was administered in doses of 25 mg. per kg. for 2 days, at which time the established subcutaneous tumor regressed almost completely. However, by the 25th day the tumor showed signs of regrowth, and cortisone was given again during the 26th and 27th days. In the ensuing 6 days, following an immediate regression, the tumors again increased in size. This process of cortisone-induced regression, followed by regrowth, was repeated four more times, the cortisone treatments being given on days 34 and 35; 42 to 45; 55 to 59; and 65 to 66. The two treatment schedules, before the last, were prolonged because the tumor failed to respond as rapidly as before. Following the treatment on the 65th and 66th day, the tumor increased in size in the presence of cortisone.

This tumor was used as the source of the resistant sublines. Two subcutaneous sublines were established; one carried in mice treated continuously with cortisone, the other carried serially in untreated mice.

TABLE 3.— *Effect of cortisone on survival time of mice bearing established subcutaneous transfers of P1798*

Transfer generation	Number of mice per group	Day begun*	Survival time (*in days*)		Percent increase in survival	
			Control avg. (*range*)	Cortisone avg.† (*range*)	From day 1	From first day cortisone begun
7	8	17	35.4(26–49)	64.6(59–69)	94.8	160
8	8	20	36.5(23–50)	56.7(33–65)	56	124
9	16	18	29.7(22–43)	48.5(20–69)	68	172

*Day of original measurement after transplantation.

†Treatments were continued until mice died. For the first 7 days the dose was 25 mg./kg. followed by a daily dosage regimen of 15 mg./kg.

A third subline was an ascitic line developed from the subcutaneously transferred line and carried in the absence of cortisone.

The responses of the resistant sublines were studied in several experiments. Experiments showing the change in tumor measurement are recorded in table 4. Cortisone was begun at two different times. In the first experiment, treatment was initiated 24 hours after tumor inoculation. In the second experiment, established subcutaneous tumors were treated with cortisone on the 20th day following tumor inoculation. Table 4 shows that tumors of the resistant subline, on the maximal dose schedule of 25 mg. per kg., increased in volume at a rate comparable to tumors in the untreated controls, regardless of the time when the cortisone treatment was started. This is in striking contrast to the response of the sensitive, established subcutaneous grafts which uniformly decreased to 1.0 percent of their original volume on the 6th day following treatment.

TABLE 4.—*Effect of cortisone on established subcutaneous transplants of the cortisone-resistant subline of P1798*

Transfer generation	Number of transfers on/off cortisone†	Percent change in tumor volume from 20th to 26th day*		
		Control	Cortisone‡	
			Begun day 1	Begun day 20
4	1/0	+212	+105	+137
5	2/0	+64	+52	+105
8	1/4	+138	+115	+102
10	1/6	+98	+143	

*Each figure represents the average of 8 mice.

†Number transfer generations carried on cortisone/number transfers carried in absence of drug. Transfer generations carried on cortisone were consecutive.

‡When cortisone was initiated 1 day after tumor inoculation, the dose was 25 mg./kg. for the first 7 days followed by 15 mg./kg. thereafter. When cortisone was begun on the 20th day, the dose throughout was 25 mg./kg.

The effect of cortisone on survival of mice bearing subcutaneous tumors from the resistant subline is recorded in table 5. In these experiments there were three groups of mice. The first was an untreated control group; the second, a group in which cortisone treatment was initiated 24 hours after tumor inoculation; and, the third, a group in which cortisone was not started until the 20th day. It may be seen that in all groups there was not a significant prolongation of life in the cortisone-treated mice. These findings again differed markedly from those of the subcutaneously passed sensitive line (tables 1 and 2 where prolongation of life was noted).

Table 6 records the effects of cortisone on the survival time of mice bearing sensitive and resistant ascites variant neoplasm P1798. The sensitive ascitic variant did not show as great an increase in survival as the subcutaneous line. This may be due to certain inherent properties of ascites tumor cells. However, the differences were appreciated when the response of the resistant ascitic variant line showed no increase in survival. The ascitic line appeared to lose some of its sensitivity as

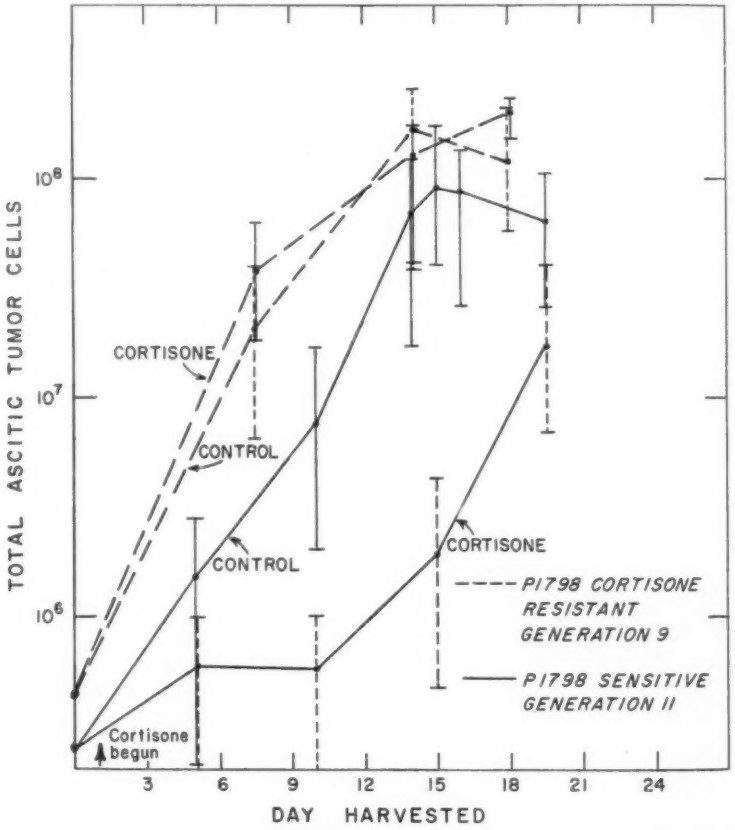
TABLE 5.—*Effect of cortisone on survival of mice bearing cortisone-resistant P1798 transplanted subcutaneously*

Transfer generation	Number of transfers on/off cortisone*	Number of mice per group†	Survival time (*in days*)		
			Control avg. (*range*)	Cortisone‡	
				Begun day 1 avg. (*range*)	Begun day 20 avg. (*range*)
4	1/0	8	30. 9(26–41)	28. 5(26–34)	31. 2(26–37)
5	2/0	8	31. 7(23–35)	31. 5(26–34)	32. 2(30–33)
6	3/0	8	32. 6(28–37)	28. 2(25–32)	
7	4/0	8	29. 5(27–31)	27. 1(18–32)	
8	1/4	8	29. 0(24–35)	32. 4(25–35)	32. 4(25–36)

*Number transfer generations carried on cortisone/number transfers carried in absence of drug. Transfer generations carried on cortisone were consecutive.

†All mice inoculated subcutaneously with 0.2 cc. of tumor brei.

‡When cortisone was initiated 1 day after tumor inoculation, the dose was 25 mg./kg. for the first 7 days followed by 15 mg./kg. thereafter. When cortisone was begun on the 20th day the dose throughout was 25 mg./kg.



TEXT-FIGURE 1.—Comparison of the effect of cortisone on growth rate of the sensitive and resistant lines of P1798. The resistant-line growth rate is more rapid than the sensitive-line growth rate.

TABLE 6.—*Effect of cortisone on survival of mice bearing sensitive or resistant P1798 ascites*

Subline	Transfer generation	Number of transfers on/off cortisone*	Cell dose ($\times 10^6$)	Number of mice per group	Survival time (*in days*)	
					Control avg. (*range*)	Cortisone avg.† (*range*)
Sensitive	6	0/0	2.2	16	22.7(20–27)	45.9(37–52) ‡
Sensitive	7	0/0	1.4	8	27.0(22–38)	36.6(31–46)
Sensitive	8	0/0	1.6	8	24.7(23–32)	33.6(32–36)
Sensitive	9	0/0	1.4	16	24.2(21–27)	30.2(23–52)
Resistant	6	2/1	2.3	8	23.5(23–24)	22.0(20–33)
Resistant	6	2/1	2.2	8	28.0(26–32)	24.8(23–26)

*Number transfer generations carried on cortisone/number transfers carried in absence of drug. Transfer generations carried on cortisone were consecutive.

†Cortisone treatment started 24 hours after inoculation of neoplasm.

‡Cortisone was not started until the 7th day after tumor inoculation.

determined by survival time when the tumor was transferred in later generations.

The effects of cortisone were more readily noted when studies were confined to the ascites population. These studies are recorded in tables 7 and 8. In these experiments the total number of tumor cells in the ascites was determined by the washing procedure previously described. Two types of experiments were performed. The first, as shown in table 7, records the total number of cells obtained when mice were started on cortisone at day 1. These findings are illustrated graphically in text-figure 1.

TABLE 7.—*Effect of cortisone on the sensitive and resistant lines of P1798 ascites: Treatment begun 24 hours after inoculation of the neoplasm*

Subline	Transfer generation	Number of transfers on/off cortisone*	Cell dose ($\times 10^6$)	Day cells harvested	Total ascites tumor-cell number ($\times 10^6$)	
					Control avg. (*range*)	Cortisone avg.† (*range*)
Sensitive	2	0/0	2.15	21	208(110–260)	4.8(1–8)
Sensitive	11	0/0	1.27	5	7.6(1–14)	3.0(0–4.8)
				10	38.2(10–75)	2.9(1–5.5)
				15	450(205–965)	9.7(2.4–21.8)
				20	311(130–540)	87.0(35–200)
				25		133(54–221)
				30		587(259–916)
Resistant	6	2/0	2.30	7	68.2(15–117)	61.3(32–106)
				14	235(110–385)	150(73–220)
Resistant	9	1/4	2.15	7	105.6(33–200)	189(95–315)
				14	867(195–1285)	637(206–832)
				18	611(285–1060)	1002(785–1175)
Resistant	11	1/6	1.67	14	703(105–1125)	826(445–1050)

*Number transfer generations carried on cortisone/number transfers carried in absence of drug. Transfer generations carried on cortisone were consecutive.

†The dose of cortisone for the first 7 days was 25 mg./kg. followed by a daily dosage regimen of 15 mg./kg.

TABLE 8.—*Effect of cortisone and prednisone on the sensitive and resistant line of P1798 ascites: Treatment started on the 14th day after inoculation of the neoplasm*

Subline	Transfer generation	Number of transfers on/off cortisone*	Cell dose ($\times 10^6$)	Day cells harvested	Total ascites tumor-cell number ($\times 10^6$)		
					Control avg. (*range*)	Cortisone avg.† (*range*)	Prednisone avg.† (*range*)
Sensitive	7	0/0	1. 47	14	505(290–1130)		
				15	791(255–1320)	89. 7(16–165)	21. 0(4–75)
				16	634(148–1092)	60. 4(6. 4–106)	20. 4(. 8–43)
				19		9. 2(2. 4–25. 7)	5. 5(. 6–15)
				24		15. 6(2. 5–31)	19. 9(6–30)
Sensitive	11	0/0	1. 27	14	351(85–615)		
				15	450(205–865)	101(44–150)	
				16	436(130–665)	76. 7(14–140)	
				19		28(3. 3–61)	
				24		12. 7(8. 6–14. 6)	
Sensitive	13	0/0	1. 70	14	434(100–775)		
				16	516(350–650)		65. 2(23–120)
Resistant	6	2/1	2. 30	14	235(110–385)		
				15	263(105–485)	280(155–550)	139. 5(87–190)
Resistant	11	1/6	1. 67	14	703(105–1125)		
				16	1116(720–1575)	1037(520–1400)	901(445–1465)
Resistant	12	1/7	2. 42	14	1343(1115–1550)		
				16	1216(750–1850)		1217(950–1715)

*Number transfer generations carried on cortisone/number transfers carried in absence of drug.
†The doses of cortisone and prednisone both were 25 mg./kg. in all experiments.

It may be seen that cortisone depresses the growth of ascites cells in the sensitive line; however, it fails to accomplish this in animals bearing resistant ascites tumors. When the study was carried over 15 days, a gradual accumulation of ascites in the cortisone-treated animals appeared. When comparison is made between the cortisone-sensitive and the cortisone-resistant populations with their respective response to cortisone between the 10th and 15th days, cortisone strikingly inhibited the sensitive population but failed to inhibit the resistant ascites. One pertinent finding regarding the cortisone-resistant line is that ascites cells accumulated at a much faster rate than the sensitive ascites even in untreated mice. This is shown in text-figure 1.

In the second type of experiment, cortisone was given to animals bearing established ascites. Results are shown in table 8. This experiment is like the type of experiment in which established subcutaneous tumors were treated (*see* table 1). A similar dramatic reduction in tumor-cell population is noted. No reduction in cell counts was seen with cortisone-resistant ascites. This effect of cortisone in reducing established ascites is illustrated in text-figure 2.

Further characterization of the sensitive and resistant population is afforded by the studies with two other compounds: prednisone and amethopterin. Amethopterin, a metabolic antagonist of folic acid, inhibits the growth of both the cortisone-sensitive and cortisone-resistant P1798 ascites sublines as shown in table 9.

TABLE 9.—*Effect of amethopterin on the sensitive and resistant P1798 ascites*

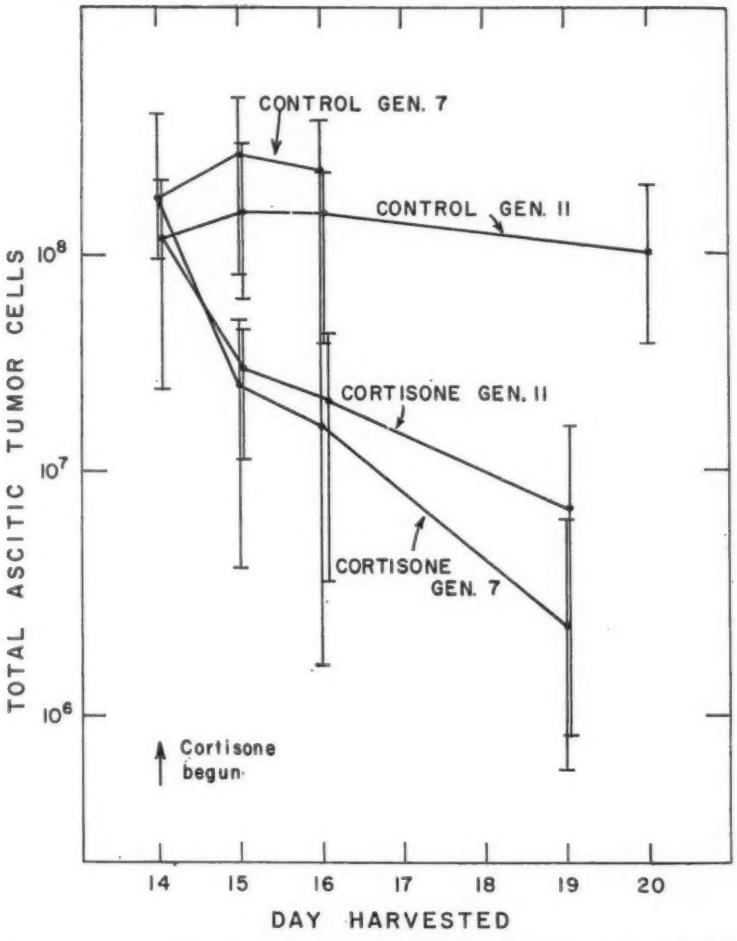
Subline	Transfer generation	Number of transfers on/off cortisone*	Number of mice per group	Cell dose ($\times 10^6$)	Day cells harvested	Total ascites tumor-cell number ($\times 10^6$)	
						Control avg. (*range*)	Amethopterin avg. †(*range*)
Sensitive	11	0/0	6	1. 27	14	351(85–615)	12. 6(7–21)
Sensitive	12	0/0	6	1. 60	14	391(21–665)	10. 1(2. 4–27)
Resistant	9	1/4	6	2. 15	14	867(195–1285)	7. 5(. 6–12. 9)
Resistant	11	1/6	6	1. 67	14	703(105–1125)	11. 6(3. 2–18. 7)

*Number transfer generations carried on cortisone/number transfers carried in absence of drug.
†Amethopterin given 2 times per week at 3 mg./kg.

Experiments comparing the activity of prednisone with that of cortisone (table 8) showed that prednisone causes a more striking regression of the P1798 sensitive line than cortisone. Furthermore, studies with the resistant subline showed a lack of response of prednisone indicating cross resistance.

Discussion

Observations reported reveal the striking growth-inhibitory effects of cortisone on the lymphosarcoma P1798. These are manifested by the suppression of growth of subcutaneously inoculated tumor, the



TEXT-FIGURE 2.—Effect of cortisone on the regression of established ascites (14th day post inoculation of neoplasm P1798). *Note* marked reduction in cell population in only 24 hours.

inhibition of ascites tumor-cell growth, and the regression of established subcutaneous and ascites-tumor populations. The effects of cortisone inhibition can be appreciated not only in growth-measurement studies but also in survival-time data in which cortisone treatment was begun 24 hours after subcutaneous tumor inoculation in the second transfer generation. None of the animals developed gross tumor and all were alive without gross tumor after 300 days. The control animals all developed tumor and were dead by the 55th day after tumor inoculation. In similar experiments repeated at later generations, but with a longer course of treatment, some tumors developed in the presence of continuous

cortisone treatment. Many of the treated animals, however, did not exhibit a progressively growing neoplasm.

The rapid regression observed in 3 to 6 days with established subcutaneous tumors, following administration of cortisone, has permitted a simple and rapid assay of the anti-tumor effects of cortisone and related substances, as well as a sensitive system that may be useful in establishing dose-response relationships. This method of assay suggests that the lymphosarcoma of the mouse may be the tumor of choice for the study of the mechanism of action of antileukemic steroids related to cortisone.

The process of development of cortisone resistance was done in a single transfer generation (*see* Results, p. 1097). P1798 resistant subline has remained resistant for 7 transfer generations in the absence of the drug. Cortisone administered in pharmacological doses (*40*) may act as a selecting agent and permit a complete change in the tumor population, from cortisone sensitivity to complete resistance.

Many points concerning cortisone sensitivity and resistance await further study. It is known that not all mouse lymphoid neoplasms are sensitive to cortisone. Furthermore, it is not known why the generalized lymphocytic leukemias of the mouse do not respond well to cortisone. One reason for this apparent lack of effect may be that neoplasms of this type have not been studied in their early transfer generations. A related question is: Does tumor progression (*39*) play a role in cortisone resistance? There is some evidence obtained from studies of the first 12 transfer generations of P1798 that there was a progressive change in the response of the neoplasm to the lower dose levels of cortisone.

There is some evidence that morphological differences exist between the sensitive and resistant lines but a close comparison of the two lines has not been completed. The differences are slight, for the resistant line retains the characteristic morphology of a lymphocytic neoplasm.

Further studies will be required to define the optimal technical method of developing cortisone resistance. Methods other than the one employed may be effective in achieving the complete selection of a cortisone-resistant population. Experiments are in progress in this laboratory to study these points.

Amethopterin profoundly inhibits the growth of the resistant as well as the sensitive line of P1798 indicating no cross resistance. However, with the cortisone-resistant subline there is cross resistance with prednisone. Furthermore, preliminary data indicate that combinations of cortisone and antimetabolites have more powerful inhibitory effects on P1798 than either one alone. Preliminary evidence indicates that 9α-fluorohydrocortisone acetate[8] is more effective than any compound yet tested in causing complete regression of established subcutaneous tumors of P1798.

The use of highly cortisone-sensitive neoplasms as the object of biochemical study appears indicated. Study of this type of neoplasm may

[8] Supplied by The Upjohn Company, Kalamazoo, Mich.

elucidate the biochemical mechanism for the destruction of normal and neoplastic lymphocytes. By a comparison of the cortisone-sensitive with the cortisone-resistant neoplasm the site or sites of action in biochemical mechanisms may be determined.

Summary

A lymphosarcoma, originating in the thymus of a diethylstilbestrol-treated BALB/c male mouse was found to be highly sensitive to cortisone through 12 transfer generations. Cortisone prolonged the life of animals bearing this neoplasm when given either immediately following tumor inoculation or after the tumor had obtained a considerable volume. When treated for only 12 days with 25 mg. per kg. of cortisone, 8 mice in the second transfer generation did not develop progressively growing tumors and they have survived for over 300 days.

Cortisone induced virtually complete regression of established subcutaneous tumors in 3 to 6 days; however, following regression, the tumors reappeared. An ascitic line has been developed which responds in the same manner as the established subcutaneous graft by undergoing profound regression following administration of cortisone.

A dose regimen of 25 mg. per kg. for the first 7 days followed by 15 mg. per kg. of cortisone, which can be given for many weeks, has been described.

Cortisone resistance has been developed by inducing incomplete regressions with daily doses of 25 mg. per kg. of cortisone and by allowing regrowth to occur each time before subsequent treatments. The cortisone-resistant line has continued to be resistant for 7 transfer generations in the absence of cortisone. Amethopterin profoundly inhibits the growth of the resistant as well as the sensitive line of the neoplasm P1798. P1798 sensitive line is very sensitive to a related steroid, prednisone, but the cortisone-resistant subline is cross resistant to this compound.

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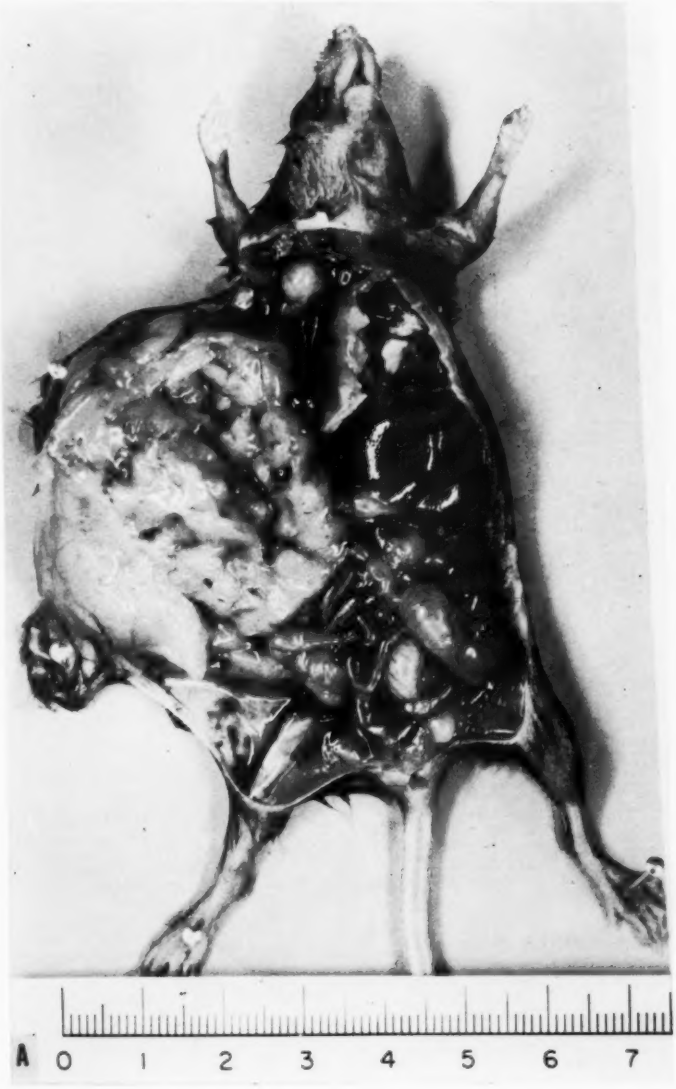
PLATE 111A

FIGURE 1A.—Control. No treatment in transfer generation 12 of P1798, 32nd day of tumor growth.



Lampkin and Potter 1109

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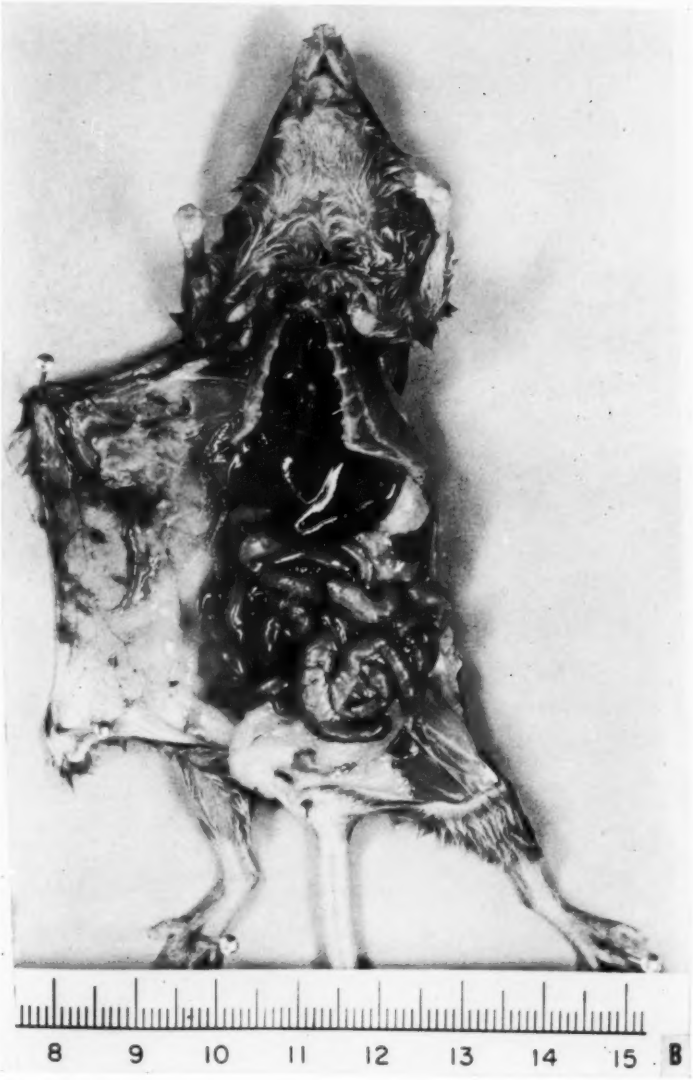
PLATE 111B

FIGURE 1B.—Cortisone treatment in transfer generation 12 of P1798 was started on the 24th day and was continued for 7 days at 25 mg. per kg. *Note* almost complete regression in the cortisone-treated mouse. Mouse was killed on 32nd day.



Lampkin and Potter

Relation of Chemical Structure to Development of Tumor Damage and Body Tremors in a Series of Phenazines and Related Compounds [1]

MARGARET G. KELLY, NORMAN H. SMITH, *and* JOSEPH LEITER, *Laboratory of Chemical Pharmacology, National Cancer Institute,[2] Bethesda, Maryland.*

A series of phenazines and structurally related compounds (derivatives and analogues) was surveyed in this laboratory for their ability to produce damage in experimental tumors in mice (*1*). Some of the compounds caused convulsions like those previously reported following toxic or lethal doses of soluble derivatives of phenazine (*2*, *3*). In addition, another striking side effect was noted. Within a few minutes after parenteral administration of certain of these compounds, mice developed severe, generalized body tremors which persisted for about an hour and were irregularly accompanied by sporadic convulsions. As many of the same compounds also induced hemorrhagic necrosis in implants of sarcoma 37, a study was undertaken to determine whether there was any relation between tumor-damaging activity and tremor-producing action. This report describes these generalized body tremors, and indicates certain correlations between chemical structure, tumor-damaging activity, and tremor production in a series of 50 compounds structurally related to phenazine.

Materials and Methods

The phenazines were injected as a suspension in 0.5 percent aqueous methyl cellulose solution; the other compounds were given in aqueous solution.

Evaluation of tumor damage was based routinely on the effect of a single intraperitoneal injection of the compound into CAF_1 mice carrying intramuscular implants of sarcoma 37.[3] The compounds were administered on the 6th day after tumor implantation and the mice were autopsied 24 hours later. The tumors were inspected for gross evidence of induced hemorrhage and sections were removed for microscopic examination of fixed tissue. A compound was rated as having "no effect" if, at the LD50, the tumor implants in the surviving mice showed no gross or microscopic evidence of induced hemorrhage and necrosis.

Survival studies were made with a few of the compounds which induced

[1] Received for publication November 22, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] For details of technique of tumor implantation, *see* Leiter *et al.* (*4*).

damage in sarcoma 37 on single injection. Mice bearing intramuscular implants of sarcoma 37, adenocarcinoma C3HBA, or leukemia L1210 were used in these experiments.

The ability of a compound to elicit tremors and convulsions was determined first in DBA/2 mice of both sexes, and then verified in CAF_1 mice carrying implants of sarcoma 37. No sex or strain differences in response were apparent. The compounds were given intraperitoneally at one-half LD50, at LD50, and at twice LD50 levels, and the mice were kept under constant observation for the first hour following injection.

The following drugs were used to study the mechanism of tremor production:

> Atropine sulfate, Merck & Co., Inc.; (2-hydroxpropyl)trimethylammonium chloride acetate (Mecholyl chloride®), Merck & Co., Inc.; sodium pentobarbital (Nembutal®), Abbott Laboratories; *N*-methyl-5-cyclohexenyl (Hexobarbital or Evipal®), Winthrop-Stearns, Inc.; chlorpromazine hydrochloride (Thorazine HCl®), Smith, Kline & French Laboratories; *N*-(2-chloroethyl)dibenzylamine (Dibenamine HCl®), Smith, Kline & French Laboratories; *d*-tubocurarine chloride (Curare or Intocostrin®), E. R. Squibb & Sons.

Results and Discussion

The series consisted of 2 piperazines, 3 pyrazinium compounds, 2 quinoxalines, 11 quinoxalinium compounds, 6 phenazines, 4 phenazinols, and 22 phenazinium compounds. The chemical name, LD50, tumor-damaging activity, tremor-producing effect, and convulsive action of the compounds used in this study are summarized in table 1. There was no apparent correlation between damage to tumor implants and production of tremors, though both activities were exhibited with greater frequency by the quaternary analogues. Practically all of the quaternary quinoxaline and phenazine compounds showed some tumor-damaging action, while only 1 of 11 quaternary quinoxalines and 11 of 22 quaternary phenazines produced generalized body tremors. Conversely, the 3 quaternary pyrazinium compounds all produced tremors, but only 1 of the group induced hemorrhagic necrosis in sarcoma 37.

Compounds were selected for this study on the basis of chemical structure, availability, and activity, from among the larger number of each class which previously had been surveyed for possible tumor-damaging effect (*1*). The proportion of tumor-damaging compounds within the different classes listed in table 1, therefore, is not intended to reflect accurately the results of the general survey, though the distribution of tumor-damaging activity is fairly representative except in the two bicyclic classes.[4] Some generalizations can be made concerning chemical structure and tumor-damaging activity. In either mono-, di-, or tricyclic classes, increased numbers of compounds exhibiting tumor-damaging

[4] Three of 25 quinoxalines and 9 of 21 quinoxalinium compounds which had been tested in the survey produced damage to sarcoma 37 at the maximum tolerated dose.

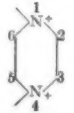
activity were found in the analogues that contained a quaternary nitrogen atom.

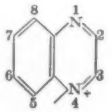
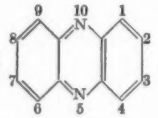
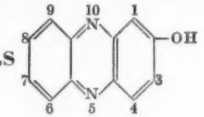
Tumor implants showed marked hemorrhagic necrosis 24 hours after a single subcutaneous, intraperitoneal, or oral injection of many of these compounds (table 1). However, areas of apparently undamaged tumor tissue could be found on microscopic examination, and the implants resumed growth after 48 to 72 hours. A few of the phenazinium compounds (NCI #3172, #2561, and #2617) also caused hemorrhage of the stomach and small intestine. Repeated injections were given of 2 quinoxalinium compounds (NCI #2410 and #2856) and of 5 phenazinium compounds (NCI #2523, #2561, #2617, #2547, and #3187) which induced severe hemorrhage and necrosis in sarcoma 37 on single injection. The compounds were administered by intraperitoneal route at LD10, LD25, and LD50 levels to mice bearing intramuscular implants of sarcoma 37, adenocarcinoma C3HBA, and leukemia L1210. In some experiments, the compounds were administered at the time the tumors were implanted and injections were continued on a weekly, tri- or biweekly schedule until death; in other experiments, treatment was not begun until 6 days after implantation. No decrease in percent of tumor "takes" was obtained, and no significant increase in survival or decrease in rate of tumor growth could be demonstrated. Gross or microscopic evaluation of induced hemorrhagic necrosis, however, was not possible on continued treatment because widespread areas of necrosis developed spontaneously by the 8th day of tumor growth.

Tremors were observed only in mice that received compounds containing a quaternary nitrogen atom (table 1). Although the quaternary nitrogen atom appeared to be essential for tremor production and also to enhance the tumor-damaging activity of the compounds, the two activities are apparently not related. As stated before, many of the compounds which induced hemorrhagic necrosis in tumor implants did not produce tremors and, conversely, several of the compounds which produced tremors did not induce tumor damage. The tremors were fine, rapid, and generalized, and were increased by voluntary movement. The tail was held erect and spasmodically swung from side to side or rotated completely. The onset of symptoms was rapid, occurring in 5 to 10 minutes after administration by subcutaneous, intraperitoneal, or oral routes, and the tremors persisted for 30 minutes to 1 hour. Tremor production appears to be independent of convulsive activity. With many of the compounds, tremors occurred at otherwise nontoxic dose levels and caused nō apparent residual effects, while convulsions were observed only at lethal levels and usually just prior to death.

The mechanism of tremor production has not been determined. However, some information on this question was obtained, indirectly, by use of drugs of known pharmacologic action on the autonomic and central nervous systems. The results of these experiments have been summarized in table 2. The pyrazinium compounds (NCI #1927, #1964, and #1965) were given to DBA mice at the LD50 level, and the phenazinium com-

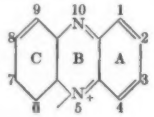
TABLE 1.—*Tremor production and tumor-damaging action of a series of phenazines, phenazine derivatives, and analogues*

NCI No.	Compound	LD50*	Effect on sarcoma 37†	Tremors‡			Convulsions		
				2 × LD50	LD50	½ × LD50	2 × LD50	LD50	½ × LD50
	PIPERAZINES 								
2094	1-(β-Chlorethyl)-4-ethylpiperazine dimethyliodide	3, 500	−	−	−	−	−	−	−
2095	1,4-bis(β-Chlorethyl)-piperazine dimethyliodide	3, 000	±	−	−	−	−	−	−
	PYRAZINIUM COMPOUNDS								
1927	1-[β-(β-Napthoyl)-ethyl]-pyrazinium bromide	450	+	+	+	+	+	−	−
1964	1-Methylpyrazinium iodide	400	−	+	+	−	+	−	−
1965	1-Ethylpyrazinium iodide	230	−	+	+	+	+	−	−
	QUINOXALINES								
2153	Quinoxaline sulfate	150	−	−	−	−	−	−	−
2931	2-Hydroxy-3,7-dimethylquinoxaline	600	+	−	−	−	−	−	−

	QUINOXALINIUM COMPOUNDS 								
2155	1-Methylquinoxalinium methylsulfate	60	+	−	−	−	−	−	−
1922	1-Ethylquinoxalinium iodide	75	+	−	−	−	+	−	−
2856	1-Propylquinoxalinium iodide	60	+	−	−	−	−	−	−
2243	1,6(7?)-Dimethylquinoxalinium iodide	75	+	−	−	−	−	−	−
2205	1-Methyl-6(7?)-chloroquinoxalinium methylsulfate	45	+	−	−	−	+	−	−
2206	1-Methyl-6(7?)-chloroquinoxalinium iodide	28	+	−	−	−	−	−	−
2410	1-Ethyl-6(7?)-chloroquinoxalinium iodide	54	+	−	−	−	−	−	−
3186	1-Methyl-6(7?)-ethoxyquinoxalinium methylsulfate	179	+	±	−	−	−	−	−
1923	1,2,3-Trimethylquinoxalinium iodide	180	+	+	+	−	+	−	−
1902	9,(11?)-Methyl-6H-indoloquinoxalinium methylsulfate	450	±	−	−	−	−	−	−
2284	9,11(?)-Dimethyl-6H-indolo-(2,3b)quinoxalinium methylsulfate	140	+	−	−	−	+	−	−
	PHENAZINES 								
1058	Phenazine	>2, 000	−			−			−
1059	2-Chlorophenazine	2, 200	−		−	−		−	−
1384	2-Methylphenazine	>600	−			−			−
1060	2-Methoxyphenazine	>1, 200	−			−			−
1308	2-Ethoxyphenazine	>2, 000	−			−			−
1608	2-Amino-8-dimethylamino-3-methylphenazine	360	±	−	−	−	−	−	−
	PHENAZINOLS 								
1507	2-Phenazinol	2, 000	±	−	−	−	−	−	−
3142	7-Chloro-2-phenazinol 5,10-dioxide	>1, 000	−			−			−
3127	8-Bromo-2-phenazinol 5,10-dioxide	>500	−			−			−
3128	8-Chloro-2-phenazinol 5,10-dioxide	1, 000	−	−	−	−	−	−	−

See footnotes at end of table.

TABLE 1.—*Tremor production and tumor-damaging action of a series of phenazines, phenazine derivatives, and analogues*—Continued

NCI No.	Compound	LD50*	Effect on sarcoma 37†	Tremors‡			Convulsions		
				2 × LD50	LD50	½ × LD50	2 × LD50	LD50	½ × LD50
	PHENAZINIUM COMPOUNDS 								
1064	5-Methylphenazinium methylsulfate	15	±	+	+	+	+	+	−
1228	5-Ethylphenazinium ethylsulfate	30	+	+	+	−	+	−	−
1968	1,5-Dimethylphenazinium methylsulfate	50	−	+	+	+	+	+	−
1644	2-Methoxy-5(?)-methylphenazinium methylsulfate	40	±	+	+	−	+	+	−
1802	2-Ethoxy-5(?)-methylphenazinium methylsulfate	55	−	+	+	+	+	+	+
3172	2-Bromo-5(?)-methylphenazinium methylsulfate	<25	+	−	−	−	−	−	−
1138	2-Chloro-5(?)-methylphenazinium methylsulfate	12	+	−	−	−	−	−	−
3187	2-Fluoro-5(?)-methylphenazinium methylsulfate	23	+	±	−	−	−	−	−
2735	2-Chloro-1,5(?)-dimethylphenazinium methylsulfate	35	±	+	+	±	+	+	±
1528	1,3-Diamino-5(?)-methylphenazinium chloride	110	+	−	−	−	−	−	−
2663	7-Chloro-1,5(?)-dimethylphenazinium methylsulfate	10	+	−	−	−	−	−	−
2662	2-Bromo-7-methoxy-5(?)-methylphenazinium methylsulfate	45	+	+	+	+	+	+	−
2523	2-Chloro-7-methoxy-5(?)-methylphenazinium methylsulfate	35	+	+	+	±	−	−	−
2618	2-Bromo-7-ethoxy-5(?)-methylphenazinium methylsulfate	75	+	+	+	−	−	−	−
2651	2-Chloro-7-ethoxy-5(?)-methylphenazinium methylsulfate	55	+	+	+	−	+	−	−
2547	2-Bromo-8-methoxy-5(?)-methylphenazinium methylsulfate	16	+	−	−	−	−	−	−
2561	2-Bromo-8-ethoxy-5(?)-methylphenazinium methylsulfate	23	+	−	−	−	−	−	−
2617	2-Chloro-8-ethoxy-5(?)-methylphenazinium methylsulfate	18	+	−	−	−	−	−	−
2955	10-Chloro-7-methylpyrido-(3,2a)-phenazinium methylsulfate	45	±	+	+	+	+	+	−
1542	3-Dimethylamino-7-amino-5-phenylphenazinium chloride	40	−	−	−	−	−	−	−
1502	2,8-Dimethyl-3-(4-dimethylaminophenazo)-7-diethylamino-5-phenylphenazinium chloride	<20	±	−	−	−	−	−	−
1543	2,8-Dimethyl-3,7-amino-5-phenyl(or tolyl)phenazinium chloride	35	−	−	−	−	−	−	−

*Expressed as μg/gm. mouse. Intraperitoneal route, 7-day observation period.

†(−) No gross or microscopic evidence of induced hemorrhage and necrosis 24 hours after single subcutaneous or intraperitoneal injection at maximum tolerated dose.
(±) Induced hemorrhage and necrosis 24 hours after single subcutaneous or intraperitoneal injection at maximum tolerated dose.
(+) Induced hemorrhage and necrosis 24 hours after single subcutaneous or intraperitoneal injection at relatively nontoxic, nonlethal dose level.

‡(−) No tremors.
(±) Mild, sporadic tremors of short duration.
(+) Severe, generalized tremors lasting 30 to 60 minutes.

TABLE 2.—*Effect of autonomic and central-nervous-system drugs on tremors produced by pyrazinium and phenazinium compounds in DBA mice**

Drug	Pharmacologic action	Dose in μg./gm. and route†	Time‡	Effect on	
				Tremors	Lethality
Atropine sulfate	Parasympatholytic	150, i.p.	15 min. *before*	No effect	Increased
Mecholyl chloride	Parasympathomimetic	50, s.c.	Immediately *before*	" "	"
Dibenamine hydrochloride	Adrenergic blockade	200, i.p.	6 hrs. *before*	" "	"
Curare	Neuromuscular blockade (skeletal muscle)	33§, s.c.	15 min. *before*	" "	"
Chlorpromazine hydrochloride	Tranquilizing, C. N. S. blockade	25, i.p.	Immediately *before*	" "	"
Hexobarbital	C. N. S. depressant, ultra short-acting	100, s.c.	10 min. *before*	" "	"
Sodium pentobarbital	C. N. S. depressant, moderately short-acting	100, s.c.	15 min. *before*	Blocked	Decreased
		100, s.c.	*After* tremors started	"	"

*Pyrazinium compounds, NCI #1927, #1964, and #1965, given at LD50 (*see* table 1).
Phenazinium compounds, NCI #2651, #2735, #2662, #1644, #1802, and #2523, given at LD50 and twice LD50 (*see* table 1).
†Intraperitoneal = i.p.; subcutaneous = s.c.
‡Expressed in relation to time of administration of pyrazinium and phenazinium compounds.
§Expressed in units × 10^{-4} per gm. mouse.

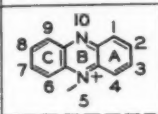
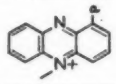
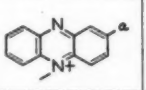
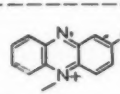
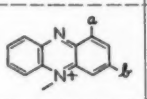
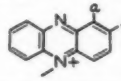
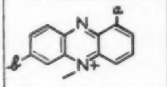
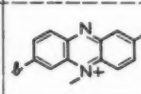
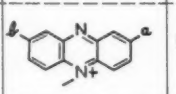
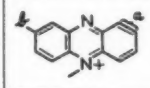
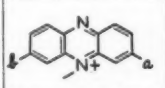
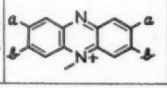
pounds (NCI #2651, #2735, #2662, #2618, #1644, #1802, and #2523) were given at doses ranging from LD50 to twice LD50. The autonomic drugs, atropine, mecholyl, dibenamine, and curare, had no measurable effect on the onset, duration, or severity of tremors, and appeared to increase the lethality of the compounds. Chlorpromazine, a tranquilizing agent, and hexobarbital, an ultra short-acting barbiturate, had no effect on tremors, and increased the lethality of the compounds. In contrast, sodium pentobarbital proved effective both in preventing development of tremors, when administered before the pyrazinium and phenazinium compounds were given, and in abolishing tremors if given after their induction. Furthermore, sodium pentobarbital gave moderate protection against the lethal effects of the tremor-producing compounds. These results suggest that the tremors may result from excitation of the central nervous system, and that they can be blocked by barbiturates of relatively strong depressant action. It is of interest that sodium pentobarbital has been reported to have a stronger hypnotic and tranquilizing effect than other barbiturates (*5*, *6*). As shown in table 2, none of the drugs had any demonstrable effect on lethality of the three phenazinium compounds (NCI #2617, #2547, and #2561) which were included for comparison because they did not induce tremors.

Compounds that did not contain a quaternary nitrogen atom (quinozalines, phenazines, and phenazinols) did not induce tremors, but neither did all of the quaternized compounds elicit this response. The simplest structure which induced tremors was that of the monocyclic pyrazinium compounds. The unsubstituted tricyclic analogue, phenazinium methyl sulfate, and about half of its substituted derivatives also exhibited this effect at relatively nontoxic doses but, in contrast, only one of the bicyclic analogues (NCI #1923) evidenced this activity, and then only at lethal levels.

Within the phenazinium group, certain correlations can be made between the structure of compounds that elicited tremors and that of compounds which did not show this property. These relationships have been summarized in text-figure 1. The position of the quaternary nitrogen in the substituted phenazinium compounds is uncertain in most cases. With the exception of the three biological stains included in the series (NCI #1542, #1502, #1543), the substituted phenazinium compounds were prepared by introduction of a quaternary nitrogen into the corresponding phenazine analogue (*7*). Theoretically, therefore, two configurations are possible for each compound, depending on whether the nitrogen in the 5 or in the 10 position in the B ring had been quaternized. In the case of the biologic stains, the method of manufacture makes possible only the configuration shown in text-figure 1 (*8*). To avoid confusion, the numbering system used in this paper assumes the quaternary nitrogen to be in the 5 position.

The tremor-producing activity exhibited by phenazinium compounds, which had no substituents in the A and C rings, was not inhibited by monosubstitution of an alkyl group at carbon 1 or of an alkoxyl group at carbon

2. In contrast, monosubstituted compounds, in which a halogen had been introduced at carbon 2, did not elicit the tremor response. This may indicate some masking or inhibiting effect of the halogen atom, or it may indicate that the halogen and alkyl or alkoxyl groups are in different positions relative to the quaternary nitrogen. Substituents in the 1,2;

TREMORS				NO TREMORS			
N.C.I. NO.	STRUCTURAL FORMULA 1/	SUBSTITUENT a	SUBSTITUENT b	N.C.I. NO.	STRUCTURAL FORMULA 1/	SUBSTITUENT a	SUBSTITUENT b
1064 1228		—	—				
1968		ALKYL	—	3172 1138 3187		HALOGEN	—
1644 1802		ALKOXYL	—	1528		AMINO	AMINO
2735		ALKYL	HALOGEN	2663		ALKYL	HALOGEN
2662 2523 2618 2651		HALO-GEN	ALKOXYL	2547 2561 2617		HALO-GEN	ALKOXYL
2955		PYRIDO	HALOGEN	1542		DI-METHYL AMINO	AMINO
				1502 1543		ALKYL	AMINO OR PHENAZO

1/ ALTERNATE NUMBERING. QUATERNARY NITROGEN IN POSITION 10.

TEXT-FIGURE 1.—Effect of structural modification on tremor production by substituted phenazinium compounds.

2,7; and 1,2,7 positions did not interfere with tremor production, but compounds with substituents in the 1,3; 2,6; 2,8; 3,7; and 2,3,7,8 positions had lost this property. With the polysubstituted phenazinium compounds, apparently the ability to elicit the tremor response is related to spatial configuration rather than to the chemical nature and number of substituent groups.

Summary

A series of 50 compounds related to phenazine (piperazines, pyrazinium compounds, quinoxalines, quinoxalinium compounds, phenazines, phenazinols, and phenazinium compounds) was studied for interrelationships between chemical structure, tumor-damaging effect, and tremor-producing activity, a previously undescribed action of some of these compounds.

The percentage of compounds which induced hemorrhagic necrosis in implants of sarcoma 37 was higher in the analogues that contained a quaternary nitrogen atom and, in general, the tumor-damaging potency appeared enhanced in these groups. However, no increase in survival was obtained with repeated injections.

Severe, generalized body tremors were induced only by quaternary compounds of the pyrazine, quinoxaline, and phenazine groups, and could be blocked or suppressed by sodium pentobarbital. The relation of configuration and tremor production by the substituted phenazinium compounds is discussed.

No direct relationship could be demonstrated between tremor production and tumor-damaging activity, though many of the compounds induced both effects.

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(*7*) Vivian, D. L.: Unpublished.

(*8*) Colour Index (Rowe, F. M., ed.), 1st ed. Bradford, Yorkshire, The Society of Dyers and Colourists, 1924.

Improved Methods for the Purification of Tumor-Localizing Antibodies[1,2]

EUGENE D. DAY, GLOVER W. BARNES, JAKOB A. PLANINSEK, *and* DAVID PRESSMAN,[3] *Department of Biochemistry Research, Roswell Park Memorial Institute, Buffalo, New York*

The methods previously employed for the purification and concentration of tumor-localizing antibodies have embodied arbitrary, empirical conditions for obtaining antibody fractions with high-localization titers in tumor tissues (30% in the Murphy-Sturm rat lymphosarcoma) and low cross-localization titers in normal tissues (*1–4*). Little attention has been paid to choosing conditions that would result in fractions containing a relatively high yield of specific tumor-localizing antibody without concomitant loss in specific antibody activity.

The methods reported here outline the conditions necessary to obtain such fractions in high yield and activity within the general framework of the absorption-elution procedures we have been using both in previous and in current work, *i.e.*, within the scheme of absorbing antibodies on insoluble tissue sediments at 37° C., and eluting them from the sediments at 60° C. The same conditions that insure these high yields of purified antibody also provide a well-controlled semiquantitative analytical basis of estimating tumor-localizing antibodies and a reproducible method of isolating them. The localization properties of preparations obtained by these procedures show a marked specificity for tumor as demonstrated by a detailed analysis of the injected rats.

These improved methods for the purification of tumor-localizing antibodies should aid considerably in our anticipated attempts to utilize tumor-localizing antibodies as carriers of diagnostic and therapeutic amounts of radioactivity specifically to human tumor sites.

Preparations

Tissues and tissue sediments.—Normal tissue sediments (the insoluble part of tissue homogenates) were prepared as described previously (*3*). Ascites Murphy lymphosarcoma sediments were prepared from ascites cells which had been largely sedimented away from red and white cells in the ascitic fluid by centrifugation [10 RCF (relative centrifugal force)

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for 15 min. at 2° C.] and by several successive washings in cold saline under the same conditions for centrifugation. Insoluble sediments of these cells were then prepared by serial homogenizations and centrifugations as previously described (*3*). Because of the instability of the lyophilized ascites-tumor sediment with respect to its capacity to absorb antibodies, storage conditions were changed from 5° C. to −20° C. A loss of as much as 75 percent of the absorption capacity of the tumor sediment has been noted over a period of 2 months when storage conditions were maintained at 5° C.

Antiserum globulins.—The anti-lymphosarcoma antiserum globulin (G-anti-ALS-pool) used for this study was obtained from the pooled antisera of 12 rabbits (20 ml. from each) which had been injected with homogenates of whole, lyophilized, ascites Murphy lymphosarcoma cells in varying amounts and by various immunization procedures (table 1). The globulin was prepared by fractionation from sodium sulfate solutions as previously described (*3*).

Portions of each individual antiserum had previously been fractionated for globulin, partially purified for tumor-localizing antibodies by our single elution procedure (*3*), and assayed *in vivo* in lymphosarcoma-bearing rats for the presence of tumor-localizing antibodies. The results (table 1) show that all antisera contained tumor-localizing antibodies and that each of the three immunization procedures was effective. Moreover, no rabbit in this laboratory (neither the 12 mentioned nor the 4 recently treated) failed to produce tumor-localizing antibodies against the ascites Murphy lymphosarcoma in at least moderate yield. It is of interest that rabbit #1043, which responded least well, and rabbit #512, which responded the best, were under the same injection protocol. Antiserum #513 was the source of antibody which had been found, after purification, to localize as high as 30 percent in the Murphy lymphosarcoma in terms of the injected dose (*4*).

Radioiodination.—The modified procedure reported in (*3*) was used throughout except as follows: the ion-exchange resin used for removing unbound iodide was changed to Amberlite® IR-45(OH), and borate-buffered saline, rather than distilled water, was used to wash the ion-exchange column after passage of the iodinated protein solution. As before, the method of equilibrium dialysis was used to check the efficiency of the resin for removing unbound iodide. It is of interest in this connection that whereas iodide is completely exchanged, iodate is not exchanged at all.

Assay for localization and determination of radioactivity.—As previously described (*3*) Wistar rats bearing the solid-ascites form of the Murphy lymphosarcoma were employed. Corrections for background localization in tissues to obtain net localization values attributable to antibody localization were made as previously described (*3, 4*) or, in some instances, by direct determination through the use of the paired, iodine-isotope label technique (*5*). Methods for determining radioactivity have remained unchanged except where the paired label technique was used.

TABLE 1.—*Production of lymphosarcoma-localizing antibodies in rabbits by various immunization procedures*

Source of globulin (*rabbit no.*)	Immunization procedure			Assay of radioiodinated antiserum globulin	
	Number of injections	Amount of lyophilized ascites-tumor cells per injection (*mg.*)	Schedule of injections and exsanguinations	Percentage of whole globulin recovered:	
				By single elution*	As tumor-localizing antibody†
512	9	100	Intraperitoneal injections of saline homogenates 3 times weekly for 3 weeks; exsanguination 1 week later.	1. 6	0. 14
513				1. 3	0. 12
1042				2. 0	0. 11
1043				1. 1	0. 05
667	9	10	As above	0. 9	0. 09
668				1. 3	0. 08
669				1. 6	0. 10
670				1. 0	0. 08
671	1	25	Intramuscular injection of cells suspended in Freund's adjuvant; exsanguination 1 month later.	1. 1	0. 07
672				1. 8	0. 10
673				1. 2	0. 11
674				0. 8	0. 08

*Radioiodinated globulin, 10 mg., absorbed on 10 mg. ascites-lymphosarcoma sediment and absorbed antibodies eluted as described (*3*).

†Eluates assayed in lymphosarcoma-bearing rats as described (*3*). Values here are given in terms of the whole globulin from which eluates were obtained rather than in terms of the percentage of injected dose.

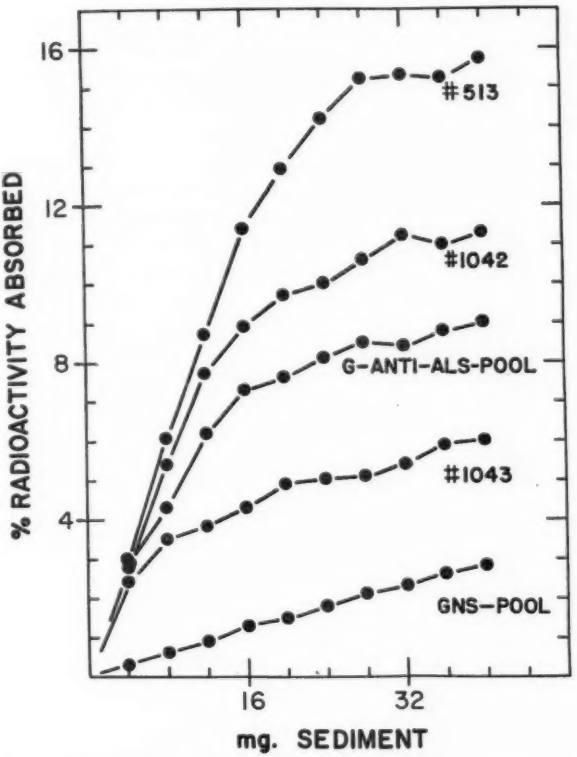
Absorption and elution.—Polyethylene 40 ml. centrifuge tubes were used during all these procedures, and a Burrell wrist-action shaker was used to provide adequate but gentle mixing of sediment and suspending fluid during all absorptions and elutions. The *p*H 8, borate-buffered saline, hereafter called borate, was drawn from a stock solution that contained 198 gm. H_3BO_3 and 150 gm. NaCl in 19.2 l. and was adjusted to *p*H 8 with 21.1 gm. of 95 percent NaOH pellets. The 50 percent rabbit serum was prepared by mixing equal volumes of borate and serum obtained from uninjected normal rabbits. The amount of radioactivity at each stage of the absorption-elution procedure was determined in the manner previously outlined (*3*).

Preliminary Observations

Antibody absorption curves.—When a constant amount of a radioiodinated anti-lymphosarcoma antiserum globulin is absorbed by varying amounts of ascites-lymphosarcoma sediment, the percentage of radioactivity absorbed, as a function of the amount of sediment used, reveals a characteristic absorption curve for each globulin (text-fig. 1). A steep rise in the amount absorbed is obtained initially, which is interpreted as absorption of antibody in a region of antibody excess. As the amount of sediment is increased further, the curve flattens. This is interpreted as additional absorption of nonspecific globulin in a region of sediment excess. In between these two extremes an optimal point can be estimated from the curves where neither the amount of globulin nor that of sediment is in appreciable excess and where the highest yield of antibody compatible with highest activity can be obtained.

In our previous studies we had arbitrarily selected amounts of sediment for absorption which often were either too small to obtain an optimal yield or too large to avoid an appreciable pickup of nonspecific globulin. In the latter case the fraction of total radioactivity attributable to antibody was lower than that at optimal conditions because of the diluting effect by large amounts of absorbed nonspecific protein. For example, the ratio of radioactivity absorbed from the pooled antiserum globulin to that from normal globulin near the optimum point (4 mg. absorbed by 16 mg. sediment) is about 7 to 1, whereas in excess sediment (4 mg. absorbed by 40 mg.) the ratio is only 3 to 1. In excess antibody (4 mg. absorbed by 8 mg.) the ratio is only slightly greater, 8 to 1, but the amount of globulin absorbed (4.8%) is only two thirds that obtainable at the optimum point.

Absorption of antibody in the presence of normal serum.—When absorptions are carried out in the presence of 50 percent normal rabbit serum (text-fig. 2), a suppression of nonspecific globulin absorption is obtained without any apparent reduction in the amount of antibody absorbed. Hence, near the optimum point (10 mg. globulin absorbed by 40 mg. sediment), a ratio of antibody to nonspecific globulin is raised from about 6 to 1 in the absence of normal serum to about 12 to 1 in the presence of normal serum. The use of serum during absorptions makes it possible to absorb antibody in high yield and yet to maintain optimal specific antibody activity in regions of antigen excess; for example, to absorb



TEXT-FIGURE 1.—Absorption of 4 mg. amounts of 4 radioiodinated rabbit anti-ascites-lymphosarcoma antiserum globulins and 1 rabbit normal-serum globulin (GNS-pool) on varying amounts of lyophilized ascites Murphy rat lymphosarcoma sediment for 1 hour at 37° C.

antibody from 10 mg. of globulin with as much as 100 mg. of sediment.

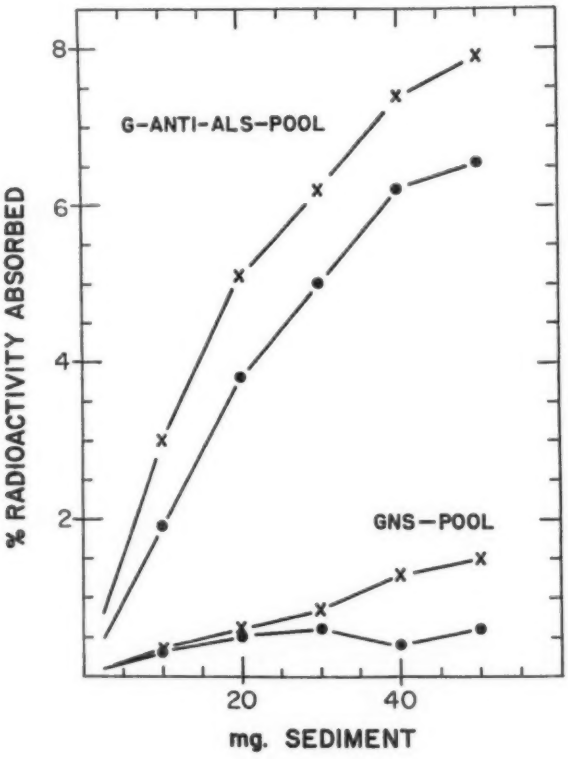
Less concentrated rabbit serum is just as effective in reducing nonspecific globulin absorptions as long as the concentration does not fall below 15 percent. More concentrated rabbit serum (95%) is no more effective. It should be noted that although the absorption of radioiodinated nonspecific globulin is reduced, it is by no means suppressed completely.

Rate of antibody absorption on sediment.—In our work on the absorption of anti-rat-kidney antibodies on rat-kidney sediment, we had observed that there was a rapid and appreciable initial absorption of antibody globulin, whereas there was no such initial burst of absorption on the part of normal globulin (text-fig. 3); thus, after ½ hour of absorption, the ratio of antiserum globulin to normal globulin was 10 to 1, whereas after 4 hours the ratio had been reduced to 4 to 1. The yield in ½ hour was only half that attained after 4 hours but could be increased by two or more short-term successive absorptions which resulted in a high com-

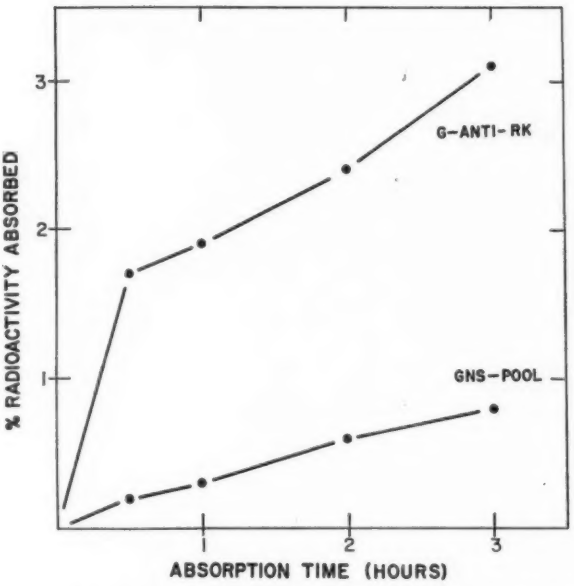
bined yield of antibody and still a low yield of nonspecific material. This principle has been found to hold also for absorptions of antibody by lymphosarcoma sediment in which two successive absorptions by half-portions of sediment for ½ hour each resulted in as high a yield as one absorption for 1 hour and with an antibody to non-antibody ratio half again as high.

Rate of antibody elution from sediment.—Whereas we have found that the rate of absorption of antibody on lymphosarcoma sediment is faster than that of non-antibody, we have found the reverse to be true for elution of tumor-localizing antibody from sediment. Here the localizing activity *in vivo* of the products eluted from sediment during the first few minutes of heat elution is lower than that of the products eluted later on.

This principle is embodied in what we have termed the *preliminary elution* procedure. After absorption of antibody upon lymphosarcoma



TEXT-FIGURE 2.—Absorption of 10 mg. amounts of radioiodinated rabbit anti-ascites-lymphosarcoma antiserum (G-anti-ALS-pool) and normal serum (GNS-pool) globulins on varying amounts of lyophilized ascites Murphy rat lymphosarcoma sediment in the presence (—●——●—) and absence (—X——X—) of normal rabbit serum, for 1 hour at 37° C.



TEXT-FIGURE 3.—Absorption of 1 mg. amounts of radioiodinated rabbit anti-rat-kidney antiserum (G-anti-RK) and normal serum (GNS-pool) globulins on 3 mg. amounts of rat-kidney sediment in the presence of 50 percent rabbit serum after varying times for absorption at 37° C.

sediment, but just prior to heat elution for antibody, much of the non-specific globulin which has been absorbed can be removed by a brief preliminary exposure of the sediment to hot borate followed by immediate centrifugation. This step, which results in a higher specific antibody activity of the subsequently eluted product, has been combined with the usual washing step following absorption. The principle is illustrated by the experiments in table 2 in which successive elutions of 5 and 15 minutes each were carried out, and by those in table 3 in which a brief preliminary elution was followed by a 20-minute elution. The assays of these eluates appear in table 4.

A lower over-all yield of radioactivity was obtained by the experiments in table 2 as compared with those in table 3 since an antibody excess (10 mg. absorbed by 20 mg. lymphosarcoma sediment) was employed in obtaining the former, and an antigen excess (10 mg. absorbed by 100 mg.), in obtaining the latter.

The total amounts of tumor-localizing antibody recovered from a unit amount of whole antiserum globulin are listed at the bottom of tables 2 and 3. These values were most easily calculated as the product of the percentage yield obtained by a given procedure and the tumor-localization activity (as fraction of injected material localizing) of the preparations assayed in rats.

TABLE 2.—*Successive elutions (5 and 15 min.) of anti-lymphosarcoma antibodies from lymphosarcoma sediment*

Fraction	Percentage of original radioactivity:		Treatment of fraction
	Run #1	Run #2	
Whole antiserum globulin	100	100	10 mg., labeled with 0.5 mc I^{131}, in 10 ml. 50% rabbit serum, absorbed with 50 mg. rat thymus sediment, 30 min., 37° C.; centrifuged 20 min., 25° C., 1500 × g to give supernatant.
Supernatant	94. 5	94. 9	Absorbed with 20 mg. ascites-lymphosarcoma sediment, 60 min. 37° C., centrifuged 20 min., 25° C., 1500 × g; sediment resuspended in 10 ml. borate and centrifuged 20 min., 25° C., 1500 × g to give washed sediment.
Washed sediment	2. 84	2. 88	Suspended in 10 ml. preheated borate; eluted at 60° C., 5 min.; rapidly cooled to 25° C.; centrifuged 20 min., 25° C., 1500 × g to give first eluate. Partially eluted sediment resuspended in 10 ml. preheated borate; eluted at 60° C., 15 min., rapidly cooled to 25° C.; centrifuged 20 min., 25° C., 1500 × g to give second eluate.
First eluate	1. 15	1. 24	Each brought to 20 ml. with 4% rabbit serum in borate for assay.
Second eluate	0. 43	0. 52	
Total eluate	*1. 58*	*1. 76*	
Recovered as lymphosarcoma-localizing antibody:			2 ml. doses of each eluate injected intravenously into 8 lymphosarcoma-bearing rats and assayed for tumor localization 18 hrs. later.
First eluate	0. 066	0. 033	
Second eluate	0. 018	0. 050	
Total eluate	*0. 084*	*0. 083*	

TABLE 3.—*Successive elutions (preliminary and 20 min.) of anti-lymphosarcoma antibodies from lymphosarcoma sediment*

Fraction	Percentage of original radioactivity:		Treatment of fraction
	Run #3	Run #4	
Whole antiserum globulin	100	100	10 mg., labeled with 0.3 mc I^{131}, in 10 ml. borate (no serum carrier), absorbed with 100 mg. ascites-lymphosarcoma sediment, 60 min., 37° C.; centrifuged 20 min., 25° C., 1500 × g; sediment resuspended in 10 ml. borate and centrifuged 20 min., 25° C., 1500 × g to give washed sediment.
Washed sediment	11. 9	12. 0	For run #3 suspended in preheated borate at 60° C.; centrifuged immediately without preliminary cooling, 20 min., 25° C. 1500 × g to give preliminary eluate. Partially eluted sediment from run #3 and uneluted sediment from run #4 suspended in preheated borate and eluted 20 min., 60° C.; centrifuged 20 min., 25° C., 1500 × g to give 20-min. eluates.
Eluted fractions:			Brought to 12 ml. with 50% rabbit serum in borate for assay.
Preliminary	2. 08	—	
20 minute	3. 40	4. 72	
Total eluate	*5. 48*	*4. 72*	
Recovered as lymphosarcoma-localizing antibody:			2-ml. doses of each eluate injected intravenously into 4 lymphosarcoma-bearing rats and assayed for tumor localization 18 hrs. later.
Preliminary eluate	0. 02	—	
20-minute eluate	0. 28	0. 28	
Total eluate	*0. 30*	*0. 28*	

Vol. 20, No. 6, June 1958
463225—58——12

TABLE 4.—In vivo *localization of fractions, obtained by successive elutions, in rats bearing the Murphy lymphosarcoma*

Fraction assayed	Run No.	Percentage of radioactivity in original whole antiserum globulin recovered:		Net localization in tumor-bearing rats (*percentage of injected radioactive dose*)				Percentage of injected dose in total blood* volume of rats
		In eluate	As tumor-localizing antibody	Tumor	Liver	Kidney	Spleen	
First eluate of table 2 (5 min.)	1	1. 15	0. 066	4. 6	0. 7	0. 0	0. 1	22
	2	1. 24	0. 033	2. 7	0. 2	0. 0	0. 2	21
Second eluate of table 2 (15 min.)	1	0. 43	0. 018	7. 2	0. 5	0. 0	0. 1	17
	2	0. 52	0. 050	9. 7	0. 6	0. 0	0. 2	23
Eluate (20 min.) without preliminary elution	4	4. 72	0. 28	6. 0	1. 0	0. 0	0. 2	20
First eluate (preliminary)	3	2. 08	0. 02	0. 9	0. 1	0. 0	0. 0	19
Eluate (20 min.)	3	3. 40	0. 28	8. 2	1. 7	0. 0	0. 3	22

*Blood volume estimated as 10% of whole rat weight.

Applications

We have shown previously (*3*, *4*) that, whereas utilization of a single absorption and elution process (single elution) results in a partial purification and concentration of lymphosarcoma-localizing antibody, successive absorption-elution steps increase the specific localization activity of antibody much further. However, final yields, usually less than 0.1 percent of the original whole globulin, have been poor.

The descriptions of the experiments which follow demonstrate how the yield of antibody obtained by double elution processes can be raised without loss in localization activity. This was accomplished by application of the principles outlined in the previous section.

The experiment outlined in table 5 utilized the principle of absorption of antibody by a near optimal amount of sediment for the first absorption. The second absorption was carried out in sediment excess with consequent high recovery not only of antibody but also of nonspecific globulin. This resulted in a product of only moderate localization activity (9.3% in terms of the injected dose). A localization activity of 20 percent had been obtained by using much less sediment for the second absorption (about 4 mg. rather than 40 mg.) but the yield in that case was much less (about 0.09%) and the fraction of original globulin localized in tumor was much lower (0.02%).

TABLE 5.—*Double elution of anti-lymphosarcoma antibodies from lymphosarcoma sediment without use of normal serum or preliminary elution*

Fraction	Percentage of original radioactivity	Treatment of fraction
Whole antiserum globulin	100	10 mg., labeled with 0.9 mc I^{131}, in 10 ml. borate (no carrier serum), absorbed with 40 mg. ascites-lymphosarcoma sediment, 60 min., 37° C.; centrifuged 20 min., 25° C., 1500 × g; sediment resuspended in 10 ml. borate, centrifuged 20 min., 25° C., 1500 × g to give washed sediment.
First washed sediment	8. 6	Suspended in 10 ml. preheated borate (no carrier serum) and eluted at 60° C., 20 min.; centrifuged 20 min., 25° C., 1500 × g to give eluate.
Eluate	3. 9	Treated as in step 1 above to give 2nd washed sediment.
Second washed sediment	0. 65	Treated as in step 2 above to give final eluate.
Final eluate	0. 39	Brought to 20 ml. with 4% rabbit serum in borate; 2 ml. doses injected intravenously into 8 lymphosarcoma-bearing rats and assayed for tumor localization 18 hrs. later.
Recovered as lymphosarcoma-localizing antibody	0. 036	

The experiment outlined in table 6 embodied all the principles for improved absorption and elution so far elucidated, as well as the principle of prior absorption with normal tissue which has previously been discussed (*3*, *4*). Here absorption with normal tissue was used to reduce cross-localizations in normal tissues and to remove some of the non-localizing anti-tumor antibodies. The latter, when present, are carried through *in vitro* absorption-elution procedures, and thereby dilute the tumor-localizing antibodies and reduce somewhat the tumor-localization values expressed in terms of percentage of injected dose. In addition, another simple but important step contributory to the high yield finally obtained was the combination of washes with supernatants after the prior absorption and the 20-minute elution steps.

The localization values of the two doubly eluted fractions described in

TABLE 6.—*Double elution of anti-lymphosarcoma antibodies from lymphosarcoma sediment in presence of normal serum and involving use of prior absorption with normal tissue, short-term multiple absorptions, and preliminary elutions*

Fraction	Percentage of original radioactivity	Treatment of fraction
Whole antiserum globulin	100	10 mg., labeled with 1.3 mc I^{131}, in 10 ml. 50% rabbit serum in borate, absorbed with 100 mg. rat liver sediment 60 min., 37° C.; centrifuged 20 min., 25° C., 1500 × g; to give supernatant. Sediment resuspended in 10 ml. 50% rabbit serum, centrifuged 20 min., 25° C., 1500 × g to give wash. Wash combined with supernatant.
Combined supernatant and wash	95. 7	Divided into 2 aliquot portions and each absorbed with 25 mg. ascites-lymphosarcoma sediment, 30 min., 37° C.; centrifuged 20 min., 25° C., 1500 × g; supernatants each reabsorbed with 25 mg. ascites-lymphosarcoma sediment, 30 min., 37° C.; centrifuged 20 min., 25° C., 1500 × g; sediments combined and suspended in 20 ml. preheated 50% rabbit serum at 60° C., and centrifuged 20 min., 25° C., 1500 × g to give preliminarily eluted sediment.
First sediment after preliminary elution	6. 4	Suspended in 10 ml. preheated 50% rabbit serum and eluted at 60° C., 20 min.; centrifuged 20 min., 25° C., 1500 × g; to give eluate; sediment resuspended in 10 ml. 50% rabbit serum and centrifuged 20 min., 25° C., 1500 × g; to give wash. Wash combined with eluate.
Combined eluate and wash	1. 5	Treated as in step 2 above to give preliminarily eluted sediment.
Second sediment after preliminary elution	0. 43	Treated as in step 3 above to give final eluate.
Final eluate	0. 24	In 20 ml., injected in 2 ml. doses intravenously into 8 lymphosarcoma-bearing rats and assayed for tumor localization 18 hrs. later.
Recovered as lymphosarcoma-localizing antibody	0. 058	

the tables are presented for comparison in table 7, together with the yields from the products and the fraction of original globulin recovered by these procedures as lymphosarcoma-localizing antibody. Included in table 7 also is a summary of results in which either the principle of absorption in the presence of normal serum was used, or that of preliminary elution, but not both. The table shows clearly what can be obtained by proper recognition of the conditions necessary for successful absorption-elution processes.

Distribution of Purified Antibody in Tumor-Bearing Rats

In order to assess the over-all disposition, *in vivo*, of such purified preparations, we have carried out a more detailed analysis of the distribution of radioactivity, in lymphosarcoma-bearing rats, of a typically purified antibody fraction obtained by our improved purification process.

Twenty mg. of the pooled rabbit antiserum globulin, prepared against lyophilized ascites Murphy lymphosarcoma cells, were labeled with 2.1 mc I^{131}, combined with an equal volume of normal rabbit serum, and divided into two equal portions. Each portion was absorbed with 100 mg. lyophilized rat-lung sediment, and the supernatants from these absorptions were purified by our double elution procedure from 40 mg. of lyophilized ascites Murphy lymphosarcoma sediment. The final eluted products, containing 0.2 percent of the original radioactivity (4.2 μc), were then combined and assayed for *in vivo* localization properties.

Each of 8 rats bearing solid ascites forms of the Murphy lymphosarcoma was injected intravenously with a 2 ml. dose of the eluate and sacrificed 20 hours later.

One ml. portions of blood were removed from each rat prior to perfusion with saline, and all other tissues were excised after perfusion for assay as indicated below. The volume of each perfusate was also measured and samples assayed for radioactivity. The contents of stomach, intestine, and urinary bladder were assayed separately.

Individual perfused tissues in 1 gram portions or less were counted for radioactivity separately, and then were combined with the remaining carcass and digested in hot 2 *N* NaOH. All dissolved except for a small residue which was assayed separately. The volume of hot alkali digest was measured and several 1 ml. volumes were assayed for radioactivity.

In this way, a detailed analysis of the distribution of radioactivity was obtained as well as an analysis of the total amount of injected radioactivity still remaining in the animals at the time of assay (table 8).

For the distribution analysis shown in text-figure 4, we divided the distribution data from table 8 into three parts (tumor, normal tissues and blood, and excreta), and estimated the fraction of each due to antibody and to nonspecific portions of the injected radioiodinated globulin.

Summary

Improvements in the purification procedures used for the isolation and concentration of tumor-localizing antibodies have been made. Underly-

TABLE 7.—In vivo *localization of fractions, obtained by various double elution procedures, in rats bearing the Murphy lymphosarcoma*

Fraction assayed	Percentage of radioactivity in original whole antiserum globulin recovered:		Net antibody localization in tumor-bearing rats *(percentage of injected radioactive dose)**				Percentage of injected dose in total blood volume of rats†
	In eluate	As tumor-localizing antibody	Tumor	Liver	Kidney	Spleen	
Double eluate obtained without use of normal serum or preliminary elution (table 5)	0. 39	0. 036	9. 3	1. 8	0. 1	0. 2	19
Double eluate obtained with use of normal serum	0. 16	0. 033	20. 9	2. 1	0. 1	0. 3	17
Double eluate obtained with use of preliminary elution	0. 21	0. 033	15. 6	1. 7	0. 1	0. 2	18
Double eluate obtained with use of normal serum and preliminary elution, and preceded by absorption with rat liver (table 6)	0. 24	0. 058	24. 2	0. 5	0. 0	0. 1	21

*Corrected for nonspecific background localization according to method in (3).
†Blood volume estimated as 10% whole rat weight.

TABLE 8.—*Average distribution of radioactivity of purified radioiodinated anti-lymphosarcoma antibodies in 8 rats bearing the Murphy lymphosarcoma*

Part of rat assayed	Weight (*gm.*)	Percentage of injected dose found:		Percentage of total unexcreted radioactivity in whole part†
		In whole part*	Per gram of part	
Murphy lymphosarcoma, perfused (complete assay)	8. 25	26. 52‡	3. 21	41. 58
Normal tissues, perfused (complete assay)				
Liver	7. 07	1. 69‡	0. 24	2. 65
Kidneys	1. 33	0. 17‡	0. 13	0. 27
Spleen	0. 80	0. 32‡	0. 40	0. 50
Lungs	1. 12	0. 16	0. 14	0. 25
Thyroid	0. 13	1. 36	10. 5	2. 13
Testes	1. 72	0. 16	0. 09	0. 25
Heart	0. 67	0. 04	0. 06	0. 06
Thymus	0. 32	0. 03	0. 09	0. 05
Brain	1. 61	0. 01	0. 01	0. 02
Subtotal	*14. 77*	*3. 94*	*0. 27*	*6. 18*
Normal tissues, perfused and washed (complete assay)				
Stomach		0. 19		
Small intestine		0. 62		
Large intestine		0. 22		
Urinary bladder		0. 00		
Subtotal	*5. 25*	*1. 03*	*0. 20*	*1. 61*
Unassimilated portions (including washes)				
Contents of:				
Stomach	0. 5	0. 99		
Small intestine	4. 9	0. 38		
Large intestine	4. 5	0. 41		
Urinary bladder	0. 3	0. 03		
Subtotal	10. 2	1. 81	0. 18	2. 84
Remaining perfused carcass				
Muscle			0. 09	
Bone			0. 24	
Skin			0. 20	
Subtotal	117. §	17. 15**	0. 15	26. 89
Rat, perfused (including tumor), subtotal	155. ‖	50. 45	0. 33	79. 10

See footnotes at end of table.

TABLE 8.—*Average distribution oa radioactivity of purified rrdioiodinated anti-lymphosarcoma antibodies in 8 rats bearing the Murphy lymphosarcoma*—Continued

Part of rat assayed	Weight (*gm.*)	Percentage of injected dose found:		Percentage of total unexcreted radioactivity in whole part†
		In whole part*	Per gram of part	
Blood perfusate (heparinized), as blood				
Washed cells	4. 5	1. 07	0. 24	
Plasma (including washes)	3. 9	12. 27	3. 15	
Subtotal	8. 4¶	13. 34	1. 59	20. 90
Rat, whole unperfused, including tumor, total	131. ‖	63. 79††	0. 49	100. 00
Eliminated from rat before assay		36. 21‡‡		

*Percentage standard deviations from these mean values for the 8 rats were: tumor, ±15%; normal tissues, ±8%; blood perfusate, ±10%; unassimilated portion, ±22%; whole rat, ±6%.

†Obtained by dividing the values for percentage of injected dose by the fraction of the dose (0.638) remaining in rats at time of assay.

‡Net localizations (total minus background localization) of antibody are: tumor, 20.8%; liver, 0.5%; kidneys, 0.0%; spleen, 0.2%.

§Obtained by difference between weight of whole perfused rat and combined weight of the subtotals above it.

‖Note that perfused weight is 20% higher than weight before perfusion since perfusion causes tissues to become edematous.

¶Volume of blood perfused was obtained by dividing radioactivity found in total perfusate by the radioactivity found in 1 ml. blood removed before perfusion.

**Obtained by difference between radioactivity found in whole perfused rat and combined radioactivity of the subtotals above it.

††Obtained by adding radioactivity found in whole perfused rat to that found in blood perfusate.

‡‡Obtained by difference between radioactivity in standard (used to determine injected dose) and radioactivity remaining in rat at time of assay.

ing these improvements are principles applicable to the absorption of rabbit anti-rat-lymphosarcoma antiserum globulins on sediments of ascites-lymphosarcoma cells and subsequent elution of the antibodies:

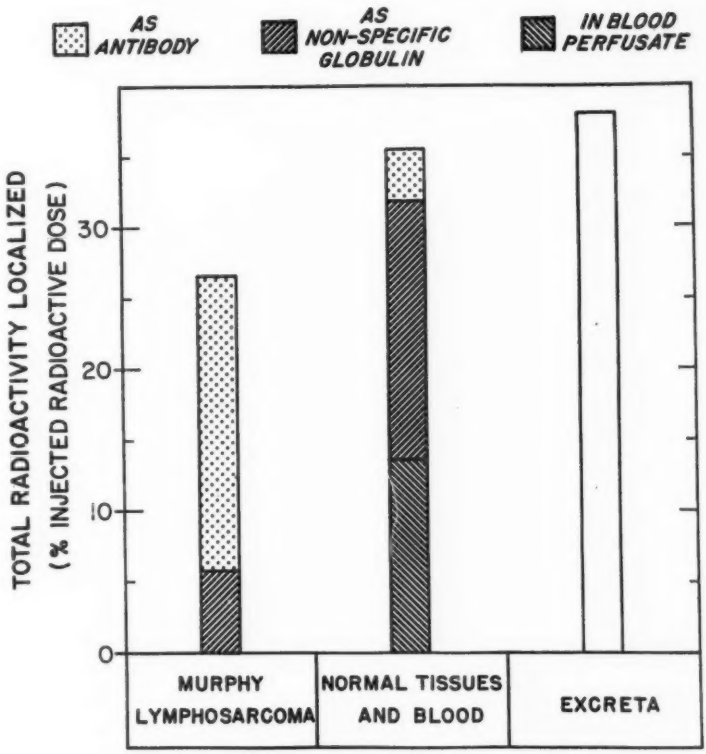
1) Absorption in regions of antibody or sediment excess leads to low yields of antibody with high specific activity or to high yields of antibody with low specific activity. Absorption in a region where neither is in excess leads to antibody in high yield and activity.

2) Absorption of radiolabeled antiserum globulins in the presence of high concentrations of normal rabbit serum reduces absorption of radiolabeled nonspecific globulin without affecting the absorption of radiolabeled antibody.

3) Absorption of radiolabeled antibody globulin proceeds at a faster rate than absorption of radiolabeled nonspecific globulin.

4) Heat elution of radiolabeled nonspecific globulin proceeds at a faster rate than that of radiolabeled antibody globulin.

A detailed analysis of the distribution of purified, radioiodinated anti-



TEXT-FIGURE 4.—Average distribution of purified radioiodinated anti-lymphosarcoma antibodies in 8 rats bearing the Murphy lymphosarcoma.

lymphosarcoma antibodies in rats bearing the Murphy lymphosarcoma was carried out to show the marked specificity of the purified antibodies for tumor.

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Immunological Studies with Nucleoproteins from Tissue-Culture Cells. I. Nucleoproteins from Human Leukemic Bone-Marrow (MCN) Cells [1]

JOHN S. COLTER, VITTORIO DEFENDI, ROSLYN E. WALLACE, *and* HARRIS H. BIRD,[2] *The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania, and Viral and Rickettsial Section, Research Division, American Cyanamid Company, Pearl River, New York*

In studies of cellular antigens, in general, and of tumor immunology, in particular, tissue-culture systems have not been used extensively. A review of the results obtained with such systems has appeared in the work of Imagawa *et al.* (*1, 2*). More recently, Mountain (*3*) reported that antiserum produced in rabbits by the intravenous injection of culture fluid in which HeLa cells had been grown and ruptured had the capacity to agglutinate and lyse HeLa cells in culture. The cytopathogenicity, in the presence of added complement, of anti-HeLa cell rabbit serum also was described by Goldstein and Myrvik (*4*). Habel (*5*) studied the cytotoxic effects of rabbit antiserum, produced by the injection of homogenates of a line of normal skin epithelial cells, on a number of cell lines maintained in tissue culture. Latta and Kutsakis (*6*) and Liu *et al.* (*7*) showed that antisera prepared in heterologous hosts against homogenates of chick-embryo heart and rat kidney inhibit the growth in tissue culture of cells derived from these organs.

Although a number of investigators in the field of tumor immunology employed particulate fractions of cells as antigens (*8–13*), little effort has been directed toward the use of antigens that could be defined either chemically or physically. Nettleship (*14*) reported that antisera produced in the rabbit by injection of protein and nucleoprotein fractions isolated from the Murphy-Sturm lymphosarcoma of rats brought about the retardation or complete regression of this tumor *in vivo*. Horn (*11*) studied the effects on growth of the Ehrlich ascites tumor of rabbit antiserum against a nucleoprotein fraction isolated from Ehrlich cell nuclei. In this laboratory (*15*), it has been shown that antisera produced in the rabbit by intravenous injection of ribonucleoprotein- or deoxyribonucleoprotein-rich fractions are capable of inhibiting the growth of this tumor in mice. Rabbit antisera have been prepared against ribonucleoprotein- and deoxyribonucleoprotein-rich fractions isolated from a strain of cells derived from human leukemic bone marrow. The effect of these antisera

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on the growth of a number of tissue-culture cell lines comprises the subject of this report.

Materials and Methods

The antigens were isolated from a line of cells, designated the MCN line, derived from the bone marrow of a young woman with chronic myeloid leukemia, by McCulloch *et al.* (*16*).[3] When used for the isolation of antigens, the cells were grown in suspended cell cultures by a technique developed in this laboratory (*17*). Cells cultivated in this way were separated from the fluid suspensions by centrifugation, washed in saline, and frozen rapidly in an ethanol-dry ice bath. The frozen cells were stored at −60 to −70° C. until a pool of 40 to 50 gm. was collected.

Ribonucleoprotein (RNP), deoxyribonucleoprotein (DNP), and the "soluble protein" fractions were isolated in the manner previously described for Ehrlich ascites carcinoma cells (*15*). The RNP fraction contained approximately 20 percent ribonucleic acid and the DNP approximately 40 percent deoxyribonucleic acid. Seven to 8 percent of the nucleotide phosphorus in the DNP fraction was ribonucleotide phosphorus. No deoxyribonucleotide phosphorus was present in the RNP fraction.

Antisera were prepared by intravenously injecting rabbits with distilled water solutions of the antigens. Each rabbit received 2 ml. of solution, containing 10 mg. antigen per ml., twice weekly for a total of 8 injections. Ten days after the final injection, the rabbits were bled out by cardiac puncture, and the sera were separated, sterilized by filtration, and stored at −25° C. Each antiserum represented a pool of sera from 3 rabbits. Normal sera were collected from the same rabbits prior to immunization.

The cell lines against which the antisera were screened for cytotoxicity were cultivated in media composed of 5 percent serum incorporated into Eagle's synthetic basal medium (*18*), modified by the use of sodium bicarbonate at 1.0 gm. per liter, and the addition of glycine, asparagine, serine, and alanine at concentrations of 0.1 m*M*. Penicillin and streptomycin, at concentrations of 250 units and 50γ per ml., respectively, were incorporated into the final mixture. In the ERK (a),[4] Detroit 52, 98, and 173, and Osgood's J-96, J-111, and J-128 lines, human placental serum was employed. Calf serum was used for the human kidney and bovine embryonic muscle lines, while rabbit serum was used for the ERK (b) line.[4] Horse serum was incorporated into the media for all other cell lines studied.

In the preparation of the cultures, cells were harvested from stock culture flasks and suspended in medium to give concentrations of 300,000 to 600,000 cells per ml. Suspensions were pipetted into the reservoir of a

[3] The cell line was made available to us through the kindness of Dr. W. Henle, Children's Hospital, Philadelphia, Penna.

[4] The ERK (a) and ERK (b) lines were brought to this country by Dr. H. Koprowski, who obtained them through the kindness of Professor Wilson Smith and Dr. J. Sheffield, of the Department of Bacteriology, University College Hospital Medical School, London, England.

glass stirring apparatus, designed like that of Evans *et al.* (*19*), and 1 ml. aliquots were delivered, under a constant flow of 5 percent CO_2 in air, to a series of culture tubes.

Culture tubes were: 16 × 150 mm. pyrex test tubes and Leighton tubes [5] of approximately the same diameter, but with a flattened area near the base to accommodate a 10 × 30 mm. coverslip. The usual procedure was to use at least 4 culture tubes for each of the antisera and for the normal rabbit serum, and to include in each group 2 tubes containing coverslips which could be removed to permit cytological and cytochemical studies.

The tubes were incubated at 37° C. in a nearly horizontal position. After approximately 24 hours, 0.1 ml. of antisera or normal rabbit serum was added to each of the tubes, giving a final concentration of antisera of 1:20. The cultures were then rotated at 37° C. in a revolving drum to insure uniform mixing of sera with culture medium.

Microscopic examinations were made at frequent intervals immediately following the addition of the rabbit sera and periodically thereafter for 48 hours. When there were cytotoxic effects, they were usually observed within the first 2 hours. Coverslip cultures from each group were taken at various intervals and were routinely fixed in Bouin's solution and stained in hematoxylin and eosin. Other fixatives and stains were used as required.

In other experiments, designed to test the potency of the antisera, cells of the MCN line were incubated as suspensions with different concentrations of antisera. Incubations were carried out in a shaking incubator at 22° C. at 150 cycles a minute. The composition of this system was as follows: 500,000 cells in the case of DNP antiserum and 700,000 cells in the case of RNP antiserum suspended in growth medium, plus required amounts of antiserum and saline solution to give a final volume of 2 ml. In later experiments it was found necessary, in order to standardize the conditions of the experiment, to add 0.2 ml. of a complement preparation of guinea-pig serum [6] to each incubation mixture. Control experiments were run with complement alone and with normal rabbit serum.

The number of viable cells remaining after various periods of incubation was determined by the method of unstained cell count (*20*). A small aliquot of the suspension was removed and mixed in a proportion of 1:5 with a 1:1,000 dilution of eosin in Tyrode's solution. The cell count was done in a hemacytometer chamber.

Results

MCN antisera and MCN cell line.—The MCN cell line, during 40 passages in this laboratory, has exhibited constant morphological characteristics (fig. 1) like those of the parent strain maintained in Dr. McCulloch's laboratory (*21*). The cells are fibroblast-like—elongated, spindle-shaped, or more often with numerous thin, delicate processes—

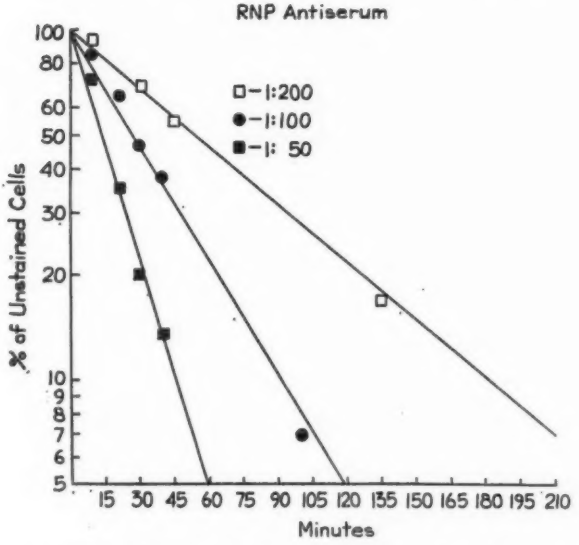
[5] Purchased from Microbiological Associates, Bethesda, Md.
[6] Vacseal Complement (Lyophilized) Carworth Laboratories, Inc., New York, N. Y.

and are organized as a network. Cell borders are always well defined, even when there is compact growth, and the nuclei are large, usually elongated, with one large nucleolus or numerous small nucleoli. In histochemical tests, the cells have exhibited moderate reductase, esterase, and xanthine oxidase activities and a high level of succinic dehydrogenase activity. Alkaline phosphatase is absent and the presence of acid phosphatase is doubtful. There is a large amount of periodic acid-Schiff positive material, both as a diffuse background and as small spherical bodies, none of which is glycogen. The Golgi apparatus is juxtanuclear, and mitochondria are rather numerous. Incubation with RNP and DNP antisera produced a series of alterations in the cells that culminated in complete cellular destruction. Detailed morphological and cytochemical descriptions of these alterations will be reported elsewhere (*22*). At this point, it is sufficient to mention that in unstained preparations, an increased granularity, rounding and swelling of the cells, increased refractivity of the nucleus, transparency of the cytoplasm, and eventually cell destruction were observed. The same morphological changes—nuclear pyknosis and loss of cytoplasm—were evident in stained preparations (fig. 2). No difference in cytopathology was noted between cells incubated with DNP and those incubated with RNP antisera.

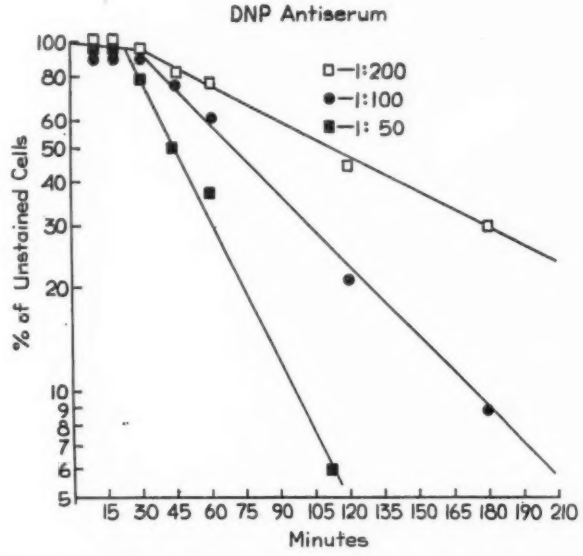
After 24 to 36 hours of incubation with the antisera, new cells were occasionally observed growing out of the destroyed ones. They also were shown to be destroyed when fresh antisera were added. No clear cytotoxicity was observed with the soluble protein-fraction antisera nor did normal rabbit serum affect the MCN strain. The cytotoxicity of the antisera was destroyed by heating for 1 hour at 56° C., but could be restored by adding guinea-pig complement or fresh normal rabbit serum to the medium. In other experiments, in which the cells were in suspension, agglutination was never observed.

The relationship between the percentage of unstained, or viable, cells and time of incubation—a measure of the potency of the antiserum—is illustrated in text-figure 1 for the RNP antiserum and text-figure 2 for the DNP antiserum. It is evident from these figures that with the DNP antiserum there is a lag period of about 30 minutes before the number of viable cells begins to decrease. After this period, the relationship between time and percentage of surviving cells becomes comparable to that shown with RNP antiserum. A lag period of the same length was also observed in the activity of DNP antiserum against the human leukocyte strain, reported in table 1. Adsorption of DNP antiserum with RNP antigen increased the duration of the lag period. With the adsorbed antiserum, no change in the percentage of unstained cells was observed during the first 40 minutes.

In other experiments, aliquots of each antiserum were adsorbed with RNP and DNP antigens at concentrations of 2 mg. per ml. for 24 hours at 5° C. The samples were then centrifuged at 20,000 r.p.m. for 1 hour, and the supernatants tested for cytotoxicity against the MCN line. Although these experiments were not altogether satisfactory, we were



TEXT-FIGURE 1.—Effect of different dilutions of RNP antiserum from the MCN cell line on the rate of cell destruction. (Suspension of 700,000 cells at 22° C.)



TEXT-FIGURE 2.—Effect of different dilutions of DNP antiserum from the MCN cell line on the rate of cell destruction. (Suspension of 500,000 cells at 22° C.)

TABLE 1.—*Cytotoxicity spectrum of MCN nucleoprotein antisera against established cell lines*

Cell Line	Ref. No.	Tissue source	Morphological characteristics*	DNP	RNP
MCN	(*16*)	Human, leukemic bone marrow	Fibroblast	+++	+++
Human leukocyte	(*21*)	Human, peripheral blood—no malignancy	Fibroblast	+++	+++
Detroit 6	(*23*)	Human, sternal marrow with metastasis of lung carcinoma	Epithelial	–	–
Detroit 52	(*24*)	Human, sternal marrow—no malignancy	Epithelial	–	–
Detroit 98	(*24*)	Human, sternal marrow—no malignancy	Epithelial	–	–
Detroit 173	(*25*)	Human, peripheral blood—no malignancy	Epithelial	–	–
J-96	(*26*)	Human, peripheral blood—subacute monocytic leukemia	Epithelial	–	–
J-111	(*26*)	Human, peripheral blood—acute monocytic leukemia	Epithelial	–	–
J-128	(*27*)	Human, peripheral blood—chronic granulocytic leukemia	Epithelial	–	–
HeLa	(*28*)	Human, cervix—epidermoid carcinoma	Epithelial	–	–
HeLa-S3	(*29*)	Clonal isolate from HeLa	Epithelial	–	–
KB	(*30*)	Human, floor of mouth—epidermoid carcinoma	Epithelial	–	–
HEP #1	(*31*)	Human, cervix—epidermoid carcinoma	Epithelial	–	–
Human kidney	(*32*)	Human kidney	Epithelial	–	–
Human liver	(*33*)	Clonal isolate from human liver (*32*)	Epithelial	–	–
Human conjunctiva	(*33*)	Clonal isolate from human conjunctiva (*32*)	Epithelial	–	–
Human appendix	(*33*)	Clonal isolate from human appendix (*32*)	Epithelial	–	–
Liver "R"	(*34*)	Human child liver with hemangioendothelioma	Epithelial	–	–
ERK (a)	(*35*)	Rabbit, embryo, normal kidney (grown in human serum)	Epithelial	–	–
ERK (b)	(*35*)	Rabbit, embryo, normal kidney (grown in rabbit serum)	Epithelial	–	–
Rabbit kidney	(*36*)	Rabbit adult normal kidney	Epithelial	–	–
14p	(*37*)	Rat, subcutaneous tissue	Fibroblast (?)	–	–
T-333	(*37*)	Malignant subline of strain 14p	Fibroblast	+++	+++
"L"	(*38*)	C3H mouse fibroblast—malignancy induced *in vitro*	Fibroblast	+++	+++
Lung To	(*34*)	Human embryonic lung	Fibroblast (?)	+++ (?)	+++ (?)
Bovine muscle	(*39*)	Bovine fetus, 2–3 months old—gluteus muscle	Fibroblast	+++	+++

*The terms "fibroblast" and "epithelial" are used only as a broad indication of the more frequently found morphological characteristics of the cell lines as they are grown in our laboratory in the culture medium specified in the methods. Cells indicated as "fibroblasts" have a cellular body, often triangular or spindle-shaped, with long, slender, cytoplasmic processes, usually 2 or more, that often anastomize with processes of proximal cells. They are associated in a network pattern. The term does not include those cells which sometimes show stretching and elongation on the glass and which have large, short processes. Cells designated as "epithelial" are those which usually have compact bodies growing in cords or sheets forming a continuous layer.

able to observe a 15- to 20-fold decrease in potency when each antiserum was adsorbed with the homologous antigen. When adsorbed with the heterologous antigen, each antiserum manifested only a 3- to 5-fold decrease. This was not unexpected in view of the fact that the deoxyribonucleoprotein fraction was contaminated with ribonucleoprotein.

MCN antisera and other cell strains.—Table 1 illustrates the results of testing the MCN antisera against a large number of cell lines. The criteria by which the cell strains were chosen were their availability in the laboratory and the possible morphological or genetic (species or tissue of origin) relation with the MCN strain. Of 25 additional cell lines tested, the "L" mouse fibrosarcoma (fig. 4), bovine embryo muscle (fig. 5), T-333 rat sarcoma (fig. 6), and human leukocyte (fig. 7) were affected by the antisera against the MCN fractions. The case of the human embryo lung is not clear, since the control cultures with normal rabbit serum showed evident signs of cytotoxicity. The degree of toxicity, as far as could be determined by observation of unstained and stained preparations, was similar in the 4 cell lines, as were the sequence and time of the morphological changes (fig. 3). The growth capacity and morphological characteristics of all other cell lines examined were completely unaffected by these antisera. In addition, fresh cells from human and monkey (*Macaca cynomolgus*) blood were tested. In neither case did the leukocytes show any alteration.

Discussion

Nucleoproteins isolated from a cell line grown in tissue culture for over 2 years have been used for the production of antisera in the rabbit. The antisera obtained showed evident cytotoxic activity like that observed by Latta *et al.* (*6*) and Flax (*40*). The cytotoxicity was not observed with normal rabbit serum, and was lost by heating. It was dependent on the presence of complement, partially eliminated by antigen adsorption, and showed some degree of specificity in its range of action. There is little doubt that we are dealing with an immunological phenomenon. That nucleoproteins are effective antigens has already been demonstrated by us (*15*) and by Horn (*11*), and there are some indications in the literature that this is also true when cellular particulate fractions are employed as antigens. Hollingsworth and Finch (*41*), for example, have suggested that microsome and supernatant fractions of leukocytes are better sources of antigens than other fractions examined. Although biochemical characterization of the cellular fractions was not done, it is highly probable that the two, more antigenic, fractions contained a large part of the cytoplasmic RNP.

While we have two different, chemically defined antigens, we do not have proof that we have two different cytotoxic antibodies. The DNP fraction is contaminated with RNP, and it is possible that RNP may be the only antigenic substance. But some considerations, in our view, suggest that we are dealing with two different antibodies. First, the titers of the two antisera do not differ significantly. It does not seem

likely that this would be true if only the RNP component were responsible for the production of antibody, as the concentration of ribonucleotide phosphorus represents only 7 to 8 percent of the total nucleotide phosphorus in the DNP fraction. Second, the adsorption experiments, though not definitive, indicated a much higher loss of cytotoxicity when the antisera were adsorbed with the homologous antigens than when they were adsorbed with the heterologous ones. And last, there is a striking difference between the two antisera in the initial portions of the curves expressing the rate of cellular destruction (text-figs. 1 and 2). This difference can only be explained on the basis of a different mode or site of action of the two antisera. At this point it is rather difficult to explain these differences. Thus, while the straight line observed with the RNP antiserum is compatible with the hypothesis that the antigen-antibody reaction is at the level of the cellular membrane, the effect of the DNP antiserum is rather puzzling. The lag period of 30 minutes with the latter antiserum could be interpreted as a reflection of the time required for the diffusion of the antibody from the cellular surface to the nucleus where the antigen is localized, or as an indication of a different and indirect mechanism of action. Preliminary studies of the sequence of cytopathological changes produced by the two antisera have thus far failed to reveal any differences.

As shown in table 1, the only cell strains apart from the MCN line affected by these antisera are the human normal leukocyte, T-333 rat sarcoma, "L" mouse fibrosarcoma, bovine muscle, and possibly the human embryo lung. A certain degree of selectivity of antisera prepared with tissue-culture antigens has been reported in the literature. In some cases, the specificity has been complete in the range tested; in others, it has been correlated with species or with embryological derivation. Thus, Imagawa *et al.* (*2*) found that antiserum produced from a mouse mammary-cancer cell line containing the mammary-tumor virus was effective against the homologous line only. Bassett *et al.* (*42*) have reported that anti-skin immune globulin is cytotoxic against ectodermal and mesodermal derived cell lines, but much less active against cells of entodermal origin. In Latta's work, (*6*) antiserum prepared against chicken fibroblasts was cytotoxic to the homologous line, but was inactive against mouse embryo heart fibroblasts. In all these studies, the range of cell lines tested was rather narrow.

In our experiments, the cell lines affected by the MCN antisera do not show any common characteristics with respect to the species or tissue of origin. Even cells, supposedly derived from a similar source, the Detroit 173 and the human leukocyte, for example, do not respond in the same way. The "L" line is a mouse fibrosarcoma whose malignant characteristics were induced *in vitro* after methylcholanthrene treatment, and the T-333 line is a rat line whose malignancy also originated *in vitro*. The human leukocyte is from the peripheral blood of an apparently healthy individual, the strain having undergone a series of changes as described by McCulloch and Parker (*43*). The bovine muscle is

from our laboratory and originated from the 7th transfer of a primary culture. The changes which occurred in this cell line are also like the ones described in the work of McCulloch *et al.* (*16*, *43*). The only common feature of the 4 cell lines affected by the MCN antisera, and a very striking one, is their complete similarity in general cellular characteristics and growth pattern. They all fall into the fibroblast category. It should be remembered that all the cell lines tested have been propagated *in vitro* for a long time—most of them for many years—and their resemblance to the original line is problematic, particularly in view of the numerous "transformations" that they have undergone.

Nevertheless, it would seem that if a cell line shows an organization pattern and cytologic characteristics that are carried on in a uniform fashion through a long series of transfers, after an initial phase of change, such a preferential pattern is intrinsic to the cell line and becomes a genetic characteristic or marker of that cell. Puck's work (*44*), in which morphological differences—epithelioid or fibroblast—between clonal strains are always maintained as constant characteristics, would support such a hypothesis.

The fact that the 14p cells are not affected by the MCN antisera, while the T-333 cells which arose from the 14p line are promptly destroyed, leads one to speculate that a qualitative change has occurred in the antigenic structure of the nucleoprotein components, which could correlate with the changes in cellular properties, such as "malignancy" and morphological characteristics. If such phenomena were correlated, it would then be possible to utilize this antiserum sensitivity as a means of differentiating between fundamental changes appearing in tissue-culture cells and changes that represent merely the response of the cells to different environmental factors.

The method utilized in this work to test the sensitivity of different cells to the MCN antisera is rather crude. It does not allow recognition of subtle differences in sensitivity among various cell lines which could be important in defining the nature of an antiserum selectivity. The unstained cell-count technique is a step forward in quantitating the antigenic relationship between different cell lines, and, therefore, in understanding some fundamental characteristics of cells in tissue culture.

Summary

Sera obtained from rabbits inoculated with nucleoproteins (DNP and RNP) isolated from a human bone-marrow leukemic strain (MCN) have been shown to be cytotoxic. The cytotoxicity is complement dependent, and the sera have been titrated with the unstained cell-count technique. In this way, a difference between the initial portions of the curves expressing the rate of cell destruction in the presence of DNP and RNP antisera has been demonstrated and interpreted as indicating a different mode or site of action of the two antisera.

The antisera were cytotoxic to only 5 of a total of 26 cell lines examined. The susceptible cells—the MCN, "L" mouse fibrosarcoma, T-333 rat

sarcoma, human leukocyte, and bovine embryo muscle lines—have in common only a marked similarity in morphological characteristics. The implications of these findings to the problems of cell characteristics and cell transformation in tissue culture have been discussed.

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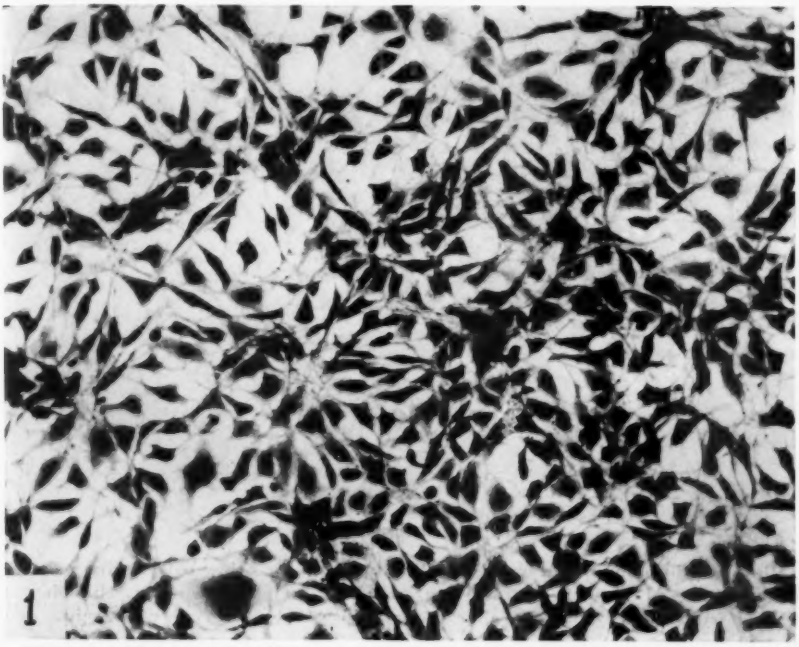
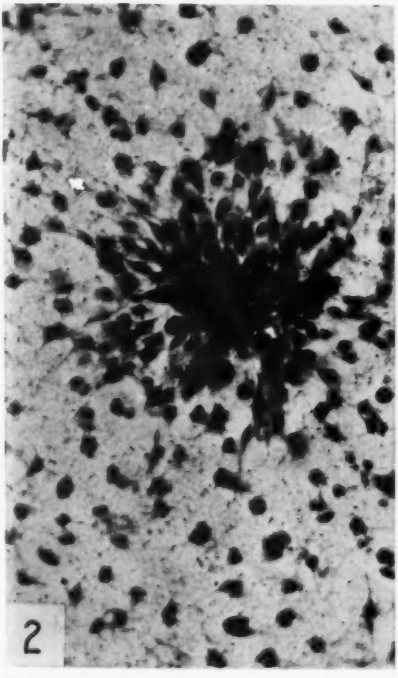
Plate 112

Figure 1.—MCN (human leukemic bone marrow) cell line—48 hours after an initial seeding of 370,000 cells in a Leighton tube. Hematoxylin and eosin. × 175

Figure 2.—MCN cell line—1 hour after addition of RNP antiserum in a dilution of 1:20. Loss of filamentous processes, rounding of cells, and the eccentric position and early degeneration of the nuclei are very evident. Hematoxylin and eosin. × 175

Figure 3.—"L" cell line—28 hours after addition of DNP antiserum in a 1:20 dilution. Disappearance of cellular contour, loss of cytoplasm, and pyknosis are manifested throughout the field. Hematoxylin and eosin. × 175





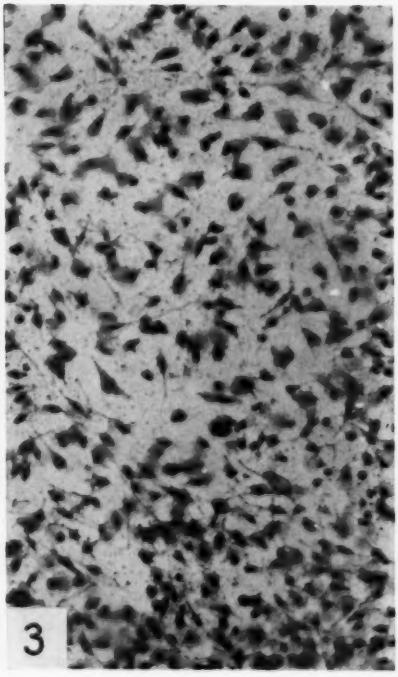


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PLATE 113

Photographs show the morphological features of the other cell strains affected by the MCN antisera. Their growth pattern is very similar, though individual cytological characteristics are evident in each cell strain.

FIGURE 4.—"L" strain. Hematoxylin and eosin. × 175

FIGURE 5.—Bovine embryo muscle. Hematoxylin and eosin. × 175

FIGURE 6.—T-333, rat sarcoma. Hematoxylin and eosin. × 175

FIGURE 7.—Human leukocyte, peripheral blood. Hematoxylin and eosin. × 175

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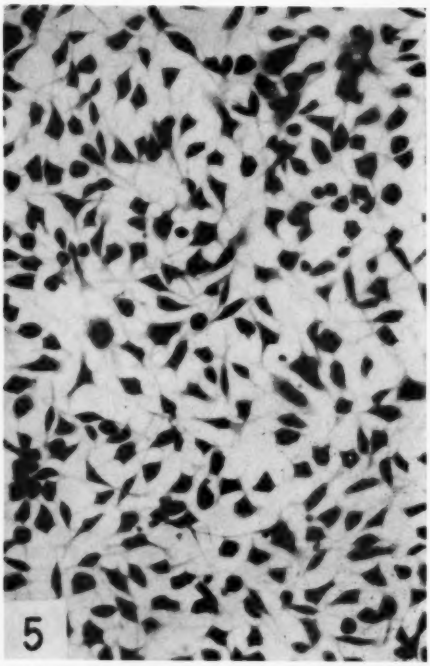
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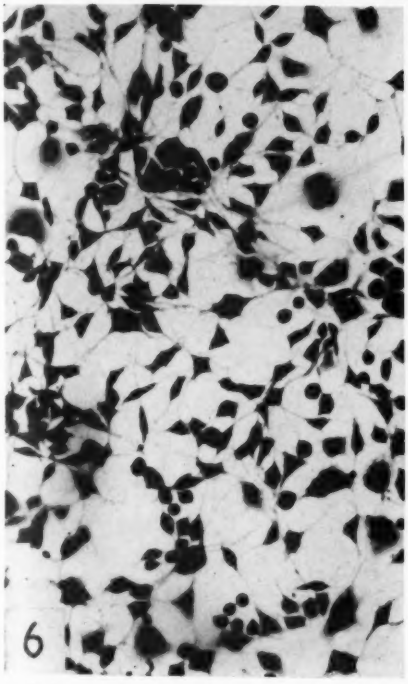
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Effect of Lethally Damaged Tumor Cells Upon the Development of Admixed Viable Cells[1,2]

László Révész,[3] *Institute for Tumor Biology and Institute for Cell Research and Genetics, Karolinska Institutet Medical School, Stockholm, Sweden*

In some previous experiments concerned with the effect of X rays on the multiplication of neoplastic cells *in vivo*, particular attention was given to the possible effect of lethally X-ray-damaged tumor cells on the growth of admixed viable cells (*1, 2*). It was found that the presence of a large number of dead or dying cells is not indifferent but may exert an important influence on the continued proliferation of the viable tumor-cell population. This finding was confirmed by Scott (*3*). It seemed of interest to carry out further investigations on the nature of this influence since mixed populations of surviving and lethally damaged cells are frequently encountered both under experimental conditions and during clinical therapy. A series of experiments has been performed and the relevant information obtained will be described.

Materials and Methods

Mice

Mice of the following inbred strains were used: C3H/StKl, DBA/HaKl, C57/HaKl (*4*), and A/Sn. Two isogenic resistant (IR) sublines of the A/Sn strain were also employed. These sublines contain different alleles at the histocompatibility-2 (*H-2*) locus but have the isogenic background of the A strain. They were developed by G. D. Snell (*5*) who sent us breeding-pairs in 1955 (*6*).

The breeding nucleus of all strains is maintained by a strict, single-line brother × sister mating. In order to maintain a high degree of homozygosis, the experimental animals are never permitted to deviate from the inbred nucleus by more than two brother × sister generations. The most distant relatives within any one strain are thus second cousins.

Special care is being taken to keep the genetic background of A/Sn strain and its isogenic-resistant sublines as closely identical as possible.

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The breeding nucleus of the IR sublines is renewed after each 5 generations of brother × sister mating. The new breeders are produced by crossing the IR line with the A/Sn strain. The resulting F_1 hybrids are crossed and mice of the F_2 generation are challenged with a tumor of strain A origin. After checking the survivors by serological typing, they are used to establish the new breeding nucleus for the next 5 generations maintained by brother × sister mating. For a number of experiments F_1 hybrids were also employed, produced by various combinations of the different basic strains.

All mice were vaccinated against ectromelia at the age of 3 to 4 weeks. A drop of vaccinia virus, propagated in tissue culture (obtained through the courtesy of Dr. Å. Espmark, of the Statens Bacteriologiska Laboratorium in Stockholm), was placed at the base of the tail and the underlying skin was scarified.

Mice of both sexes at 2 to 3 months of age were used for the experiments. Within each experiment mice of similar weight and sex were selected, unless noted otherwise. They were kept on a standard pellet diet that, with the drinking water, was available *ad libitum*.

Tumors

The following main groups of tumors were used:

1) Spontaneous mammary carcinomas that arose in females of the breeding nucleus of strains C3H, DBA, and A. Tumors were selected that showed no ulceration and that had diameters varying between 1.5 to 3 cm.

2) Sarcomas were induced in A.SW mice or in F_1 hybrids, produced by crossing the A strain with its IR-subline, A.SW. One mg. methylcholanthrene, dissolved in 0.1 ml. trioctanoin, was deposited into the musculature of the right thigh of 1-month-old animals. The 5 sarcomas used were designated MWA (originated in an A.SW mouse), MSWB, DSWB, MNSB, and MSWC, all originating in different (A × A.SW)F_1 hybrids. The transplantability of the 4 latter tumors in various isogenic-resistant mice had been studied earlier (*6*). Histologically they were polymorphous spindle-cell sarcomas.

3) The DBA ascites lymphoma originated in the thymus gland of a DBA mouse at the National Cancer Institute in 1947 (*7*). The line used was converted to the ascites form in 1950 (*8*) and has been maintained since then by weekly ascites transfers in DBA mice or by frozen storage at −79° C. in a tumor bank (*9*). The growth characteristics of this neoplasm have been studied previously (*8*, *10*).

4) Two lines of the Ehrlich carcinoma were used. They will be referred to as the Ehrlich/Sto and ELD/Sto lines, respectively. The exact time of origin of these lines is unknown. Probably they have no common origin and trace back to the various mammary carcinomas that originated in heterozygous mice in Ehrlich's laboratory around the turn of the century. Detailed studies on the growth characteristics of the particular lines used have been published earlier (*11*, *12*).

With the exception of the Ehrlich tumors and the DBA ascites lymphoma, all other tumors were used after a single transfer generation or very few (less than 5) transfer generations after their origin. These transfers were made exclusively within the host genotype of origin. The use of "young" tumors was considered important in order to minimize, as far as possible, the accumulation of immunogenetical differences between the tumor cells and their hosts. Accumulation of serial transfer generations was avoided by the frequent use of new spontaneous or induced tumors and by additional help from the frozen tumor bank in which several parallel tubes from the original tumor or its very first transfer generation were stored. The method of freezing and thawing has been published previously (*9*). No evidence of any change in immunogenetic specificity or the operation of any selection pressure was found to result from this procedure.

The ascites tumors were used in their natural suspension form. Solid tumors, aseptically removed, were freed from macroscopically visible necrotic areas. The cells were brought into suspension by pressing them through a 60-mesh, stainless-steel screen into Ringer's solution, which contained penicillin and streptomycin in concentrations of 100 IU and 100γ per ml., respectively. The proportions of tissue and suspending fluid were chosen so that 1 ml. contained about 0.3 ml. wet tissue pulp. The suspended cells were counted in a hemocytometer and appropriate dilutions were made with Ringer's solution. Schrek's method of unstained-cell counts (*13*) was used to estimate the approximate proportion of viable cells. This estimate was considered as a probable maximal limit of the viable count. Because of the uncertainty of the real viable count, comparisons were restricted to different dilutions of the same original cell suspension within each experimental series. All suspensions were kept in an ice-bath before inoculation. The injections were completed within 1 hour after the dilutions were made. Fluid volumes of 0.1 to 0.2 ml. were deposited into the right flank of the mice with a tuberculin syringe and a 23-gauge needle, unless stated otherwise.

The inoculation site was checked by regular palpations and the survival time of the tumor-bearing animals was recorded. Tumor diameters were measured by caliper in a number of experiments. The observation period varied in the different experiments between 2 and 16 months (*see* Results).

Irradiation

X rays were generated in a Siemens' Stabilivolt unit at 155 kv., 6 ma., filtered by 0.1 Cu + 1 Al, HVL was 0.14 mm. Cu.

Irradiation of the ice-cooled tumor-cell suspensions was performed *in vitro* in an irradiation chamber containing 6 to 10 ml. of fluid. The details of the chamber have been described previously (*1*). With the exception of some early experiments in which cell suspensions were irradiated in an N_2 atmosphere, all irradiations took place in air. The gas was led through a gas-washer filled with water and its streaming kept the

cell suspension in constant motion during irradiation. The distance between the focus and the bottom of the irradiation chamber was 18.5 cm. About 2 mm. from the bottom, the dose rate was 460 r per minute as measured by a wavelength independent ionization-chamber.

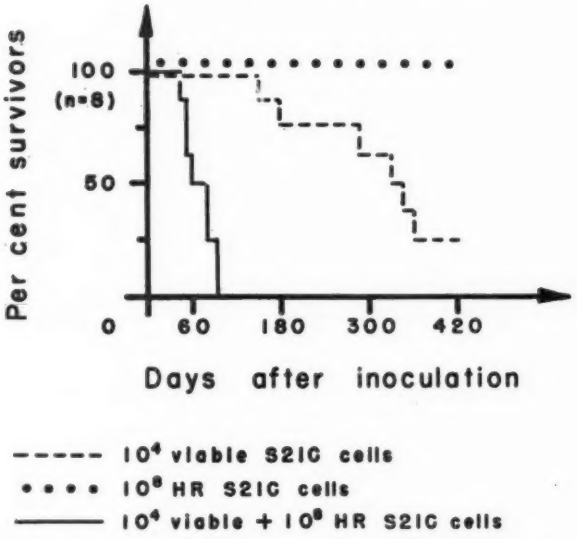
A brass tube, about 0.5 mm. thick, with a height of 77 mm. and a diameter of 28 mm., covered on the inside with a lead shield of 2 mm. thickness was used for the irradiation of tumors or of normal legs *in vivo*. On one end, the tube was attached to a device that fitted the roentgen tube in a well-defined position. A methacrylate polymer plate could be attached to the other end, which served as the bottom of the tube. Together with a semicircular hole, which was cut out from the lateral wall of the tube, this formed an opening of 1.5 cm. in diameter. The size of the opening could be regulated by inserting 2-mm.-thick lead diaphragms of different sizes. The right leg or well-circumscribed tumors of the right flank were kept in position by the appropriate diaphragm and by attachment of the floor. The rest of the body was placed outside the tube on the plastic floor. During the procedure, the mice were anesthetized by sodium pentobarbital (Nembutal®) 1 mg. per 20 gm., and immobilized by rubber bands attached to the feet. The focus-skin distance was about 13.5 cm. The dosage at this position was 1100 r per minute. It was measured with ionization chambers within a phantom corresponding to the position of the tumor. The phantom of a "tissue equivalent" material was prepared according to Jones and Raine (*14*).

Results

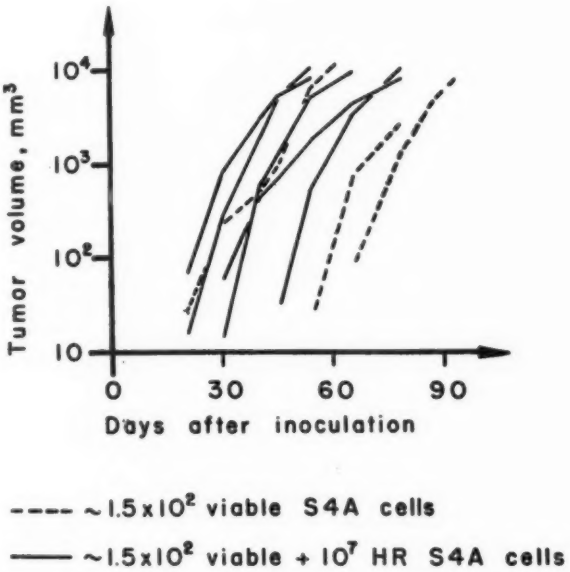
Heavily Irradiated Cells and Admixed Viable Cells

Table 1 gives a survey of all pertinent experiments. Viable tumor cells or mixtures of viable and HR (heavily irradiated) cells of the same kind were inoculated subcutaneously into genetically compatible mice. Ten different carcinomas, 3 sarcomas, and 1 lymphoma were tested. The findings uniformly indicate that the small viable inoculum received an intense stimulus for proliferation whenever HR cells were present. This was manifested by the higher incidence of progressively growing tumors and the shorter survival time in the mice inoculated with a mixture of viable and HR cells (*see* table 1, cols. 11, 14, 16) when compared to the controls that received the same number of viable cells alone (*see* cols. 10, 13, 15). In text-figure 1 the results of experiment 1*c* are illustrated in more detail.

In two experiments (6 and 7 *in* table 1) the increase of the subcutaneously growing tumors was followed by regular caliper measurements on individual mice beginning 3 weeks after inoculation and proceeding until the ulceration of the skin. Three different diameters were measured and their geometrical mean was calculated. The approximate tumor volume was roughly estimated by calculating the volume of a sphere from the mean diameter. The findings in one of the two experiments are illustrated in text-figure 2. The growth rate of the individual tumors did not show



TEXT-FIGURE 1.—Tumor incidence and survival of mice after the subcutaneous inoculation of 10^4 viable and 10^8 HR cells of the S21C tumor, alone or in mixture, respectively. (n = Number of mice inoculated in the different groups.)



TEXT-FIGURE 2.—Growth of the S4A carcinoma, estimated from caliper measurements performed on individual animals inoculated subcutaneously with 1.5×10^2 tumor cells alone or with mixtures of 1.5×10^2 viable and 10^7 HR cells, respectively.

TABLE 1.—*Tumor incidence and survival time in genetically compatible hosts after the inoculation of viable tumor cells alone or in mixture with heavily irradiated (HR) cells of the same kind*

Experiment	Tumor				Conditions of inoculation			Observation period (*days*)	Proportion of mice with progressively growing tumors*			Range of survival time of mice killed by tumors (*days*)		Median survival time (*days*)	
	Designation	Type	Transplant generation	Mouse strain of origin	Mouse strain inoculated	Number of implanted viable cells	Number of implanted HR cells (*X-ray dose: 12–15,000 r*)		Viable cells alone	Viable + HR cells	HR cells alone	Viable cells alone	Viable + HR cells	Viable cells alone	Viable + HR cells
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 a	S21C	Sp. mammary carcinoma.	1st	C3H ♀	C3H ♀	$\sim 10^4$	$\sim 10^8$	90	0/10	8/8	0/8	—	62–72	>90	65
b	"	"	"	"	(A × C3H) F_1 ♀	"	"	90	2/10	8/8		61–73	56–78	>90	62
c	"	"	"	"	(C57×C3H) F_1 ♀	"	"	90 420	0/8 6/8	8/8		— 193–360	30–76	>90 330	64
2	S22C	Sp. mammary carcinoma.	1st	C3H ♀	C3H ♂	$\sim 5 \times 10^3$	$\sim 10^9$	175	3/10	10/10	0/4	93–151	49–131	>175	69
3 a	S23C	Sp. mammary carcinoma.	1st	C3H ♀	C3H ♂	$\sim 1.7 \times 10^4$	$\sim 10^7$	137	10/10	10/10	0/8	67–137	32–66	90	52
b	"	"	"	"	(A × C3H) F_1 ♂	"	"	65		10/10			38–65		42
c	"	"	"	"	(C57×C3H) F_1 ♂	"	"	79		10/10			40–79		53
4	MSWB	MC-induced sarcoma.†	3rd	(A × A.SW) F_1 ♂	(A × A.SW) F_1 ♂	$\sim 10^4$	$\sim 10^7$	98	8/10	10/10	0/3	50–86	26–81	83	61
5 a	S24C	Sp. mammary carcinoma.	1st	C3H ♀	C3H ♂	$\sim 5 \times 10^3$	$\sim 10^7$	100	4/4	4/4	0/15	69–100	29–69	80	59
b	"	"	"	"	"	$\sim 5 \times 10^2$	"	120	3/4	4/4		98–119	64–84	102	64

c	"	"	"	"	"	~5 × 10^1	"	120	0/4	4/4		—	72–92	>120	85
6	S4A	Sp. mammary carcinoma.	4th	A ♀	(A × A.SW) F_1 ♂	~1.5 × 10^2	~10^7	119	3/5	5/5	0/3	75–106	60–119	106	72
7	MNSB	MC-induced sarcoma.†	4th	(A × A.SW) F_1 ♂	(A × A.SW) F_1 ♂	~3 × 10^3	~10^6	102	1/4	4/4	0/2	90	51–100	>102	55
8	S25C	Sp. mammary carcinoma.	1st	C3H ♀	C3H ♂	~3×10^5	~10^7	81	5/5	5/5	0/2	54–81	42–56	61	49
9	MWA	MC-induced sarcoma†	2nd	A.SW ♂	(A × A.SW) F_1 ♂	~10^5	~5 × 10^7	127	4/4	4/4	0/2	72–116	44–74	74	44
10 a	MWA	MC-induced sarcoma†	3rd	A.SW ♂	A.SW ♀	~2 × 10^3	~10^6	120	0/5	4/5	0/2	—	44–64	>120	59
b	"	"	"	"	A.SW ♀	~10^5	"	"	4/5	6/6		42–90	34–58	50	46
11 a	MWA	MC-induced sarcoma†	2nd	A.SW ♂	A.SW ♂	~1.5 × 10^3	~10^7	105	3/4	4/4	0/4	58–91	46–63	62	51
b	"	"	"	"	A.SW ♂	10^1	"	"	0/4	4/4		—	52–69	>105	52
c	"	"	"	"	A.SW ♀	~1.5 × 10^3	"	"	1/4	4/4		69	37–95	>105	41
12	S11S	Sp. mammary carcinoma.	1st	A ♀	(A × A.SW) F_1 ♂	~10^1	~10^7	120	4/10	10/10	0/5	75–98	44–70	>120	58
13	S26C	Sp. mammary carcinoma.	1st	C3H ♀	(C3H × DBA) F_1 ♂	~10^3	~10^6	160	3/7	7/7	0/2	51–126	40–62	>160	52
14	S27C	Sp. mammary carcinoma.	1st	C3H ♀	C3H ♂	~10^3	~10^7	226	3/7	7/7	0/3	111–180	68–105	>226	93
15	S28C	Sp. mammary carcinoma.	1st	C3H ♀	(C3H × DBA) F_1 ♂	~2× 10^3	~10^6	176	3/4	5/5	0/2	94–132	44–128	118	60
16 a	DBA lymphoma	Transplanted lymphoma	>400	DBA	A × DBA ♀	~4	~2 × 10^8	90	0/6	3/6	0/4		36–55	>90	55
b	"	"	"	"	"	~40	"	"	1/6	6/6		59	33–58	>90	39

*Expressed as the ratio of mice killed by progressively growing tumors and the total number inoculated.
†Induced by the intramuscular injection of 1 mg. methylcholanthrene.

Vol. 20, No. 6, June 1958
463225—58——16

major differences between the group originating from viable cells alone and the animals inoculated with mixtures of HR and viable cells. The presence of HR cells appears to reduce the latency period but does not seem to influence the growth rate of established tumors.

As shown by experiments 5, 10, 11, and 16 of table 1, the stimulating effect of HR cells was most apparent when the proportion of admixed viable cells was low.

The interpretation of all experiments is based on the assumption that heavily irradiated cells were prevented from continued reproduction. This assumption was tested in all experiments by inoculating a group of control mice with large numbers of HR cells alone. As shown in column 12, table 1 and column 11, table 4, none of these animals developed tumors during the observation period; nor was there any tumor development noted at the site of HR inocula in other experiments, reported later in this paper, in which the mice were carrying a progressively growing tumor in another region of the body. This justifies the conclusion that the X-ray dose, delivered to the HR cells, was sufficiently high to prevent their continued reproduction completely and regularly.

Heavily Irradiated Cells and Admixed Cells with Reversible Radiation Damage

The influence of HR cells on the growth of admixed, sublethally X-ray-damaged tumor cells was studied in two separate experiments. In the first experiment, 3×10^5 cells of a spontaneous mammary carcinoma of a C3H female mouse (designated S25C) were inoculated subcutaneously. The cells were either untreated or received 200 or 400 r *in vitro* prior to implantation. Parallel groups of mice received similar inocula, which, in addition, contained 10^7 HR cells derived from the same tumor. The

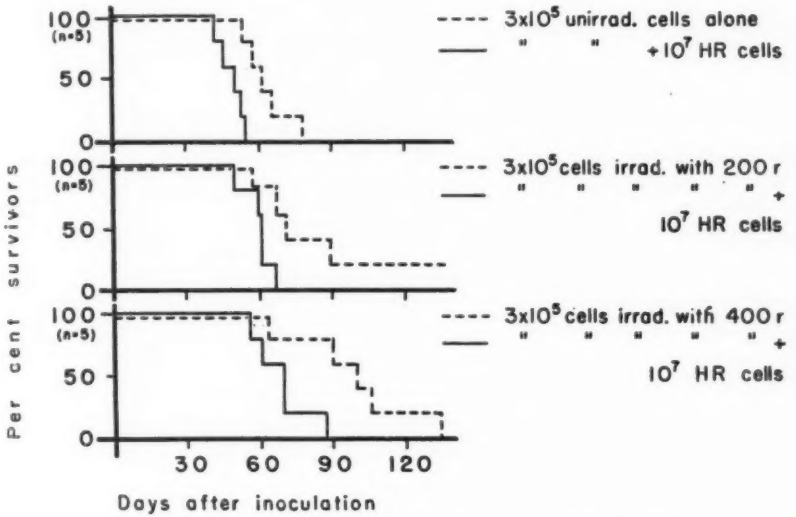
TABLE 2.—*Incidence of tumors after the inoculation of 10^7 irradiated S29C carcinoma cells alone or in mixture with 10^7 HR cells, preirradiated with 15,000 r*

X-ray dose (r)	Tumor incidence*	
	Irradiated cells alone	Irradiated cells + HR cells
500	5/5	5/5
700	5/5	5/5
1000	5/5	5/5
1400	1/5	5/5
2000	0/5	1/5
2800	0/4	0/5

*Number of animals killed by progressively growing tumors/total number inoculated.

results are illustrated in text-figure 3. Sublethal X irradiation has led to a noticeable prolongation of the survival time. This was partially counteracted by the presence of HR cells, however.

In the second experiment, the suspension prepared from another spontaneous C3H carcinoma (designated S29C) was irradiated with various X-ray doses from 500 to 2800 r. One hour after irradiation, each suspension in doses of 10^7 cells was inoculated into different groups of $(A \times C3H)F_1$ hybrid mice, either alone or after having been mixed with 10^7 HR cells of the same tumor which had previously received an X-ray dose of 15,000 r. The results are shown in table 2. There is some indication that the incidence of progressively growing tumors increased in some groups in which HR cells were present. Concurrently, there was also a reduction of the survival time.

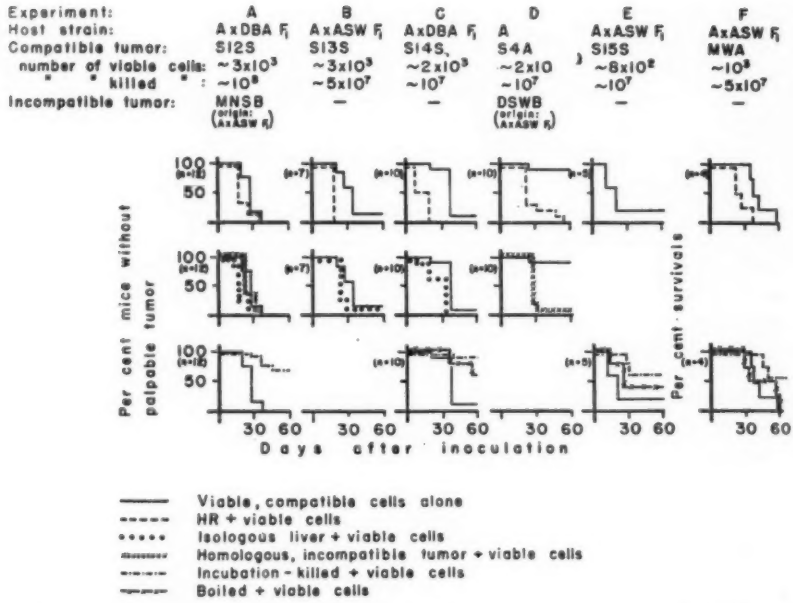


TEXT-FIGURE 3.—Survival of C3H mice after the inoculation of 3×10^5 unirradiated or sublethally X-ray-damaged suspensions of mammary carcinoma S25C. The cells were inoculated either alone or in a mixture containing about 10^7 HR cells from the same tumor pretreated with 15,000 r.

Effect of Cells Killed by Heat or Prolonged Incubation

In four different experiments (expts. A, C, E, and F of text-fig. 4) about 10^3 viable cells of the spontaneous mammary carcinomas S12S, S14S, and S15S of strain A origin and of the methylcholanthrene-induced sarcoma MWA (originated in A.SW) were inoculated into genetically compatible mice, alone or in mixture with 10^7 to 10^8 cells of the same tumor killed by incubation or by heat. A separate group of mice was inoculated with viable and HR cells in the same proportions.

Incubation of the tumor-cell suspensions was carried out in a water-bath at 37° C. during a period of 24 hours. Heat-killed cells were pre-


Experiment: A B C D E F
Host strain: AxDBA F1 AxASW F1 AxDBA F1 A AxASW F1 AxASW F1
Compatible tumor: S12S S13S S14S, S4A S15S MWA
number of viable cells: ~3x10³ ~3x10³ ~2x10³ ~2x10 ~8x10² ~10³
" " killed " : ~10⁶ ~5x10⁷ ~10⁷ ~10⁷ ~10⁷ ~5x10⁷
Incompatible tumor: MNSB (origin: AxASW F1) — — DSWB (origin: AxASW F1) — —
Per cent mice without palpable tumor
Per cent survivals
100
50
30 60
Days after inoculation
Viable, compatible cells alone
HR + viable cells
Isologous liver + viable cells
Homologous, incompatible tumor + viable cells
Incubation - killed + viable cells
Boiled + viable cells

TEXT-FIGURE 4.—Tumor incidence and survival of mice after the inoculation of viable, compatible tumor cells alone or in mixture with one of the following additional components: HR, incubation-killed or boiled tumor cells of the same kind, isologous liver cells, or homologous, incompatible tumor cells.

pared by immersing the test tube containing the cell suspension into boiling water for 10 minutes. The efficiency of these procedures was controlled by inoculating the treated cells into groups of 3 to 7 mice in all experiments. No takes were obtained in a total of 32 mice during an observation period of 2 to 3 months.

A comparison of the incidence of tumors (expts. A, C, and E) and of the survival of tumor-bearing mice (expt. F) illustrated in text-figure 4 indicates that the admixture of incubation-killed or boiled cells may inhibit the proliferation of a small viable inoculum in contrast to the simultaneously observed stimulating influence of HR cells.

Effect of Isologous Liver and Genetically Incompatible Tumor Cells

In four experiments (A to D, text-fig. 4) small viable inocula (varying between 10 and 10^3 cells) derived from 1 of the 4 carcinomas S12S, S13S, S14S, and S4A of strain A origin were inoculated into genetically compatible mice. The cells were tested alone and in mixture with viable liver cells from the same mouse genotype (isologous liver cells), or viable but genetically incompatible MNSB and DSWB methylcholanthrene-induced sarcoma cells derived from a foreign genotype and shown to be unable to grow progressively in A mice. Both isologous liver cells and incompatible tumor cells can be regarded as viable and metabolically

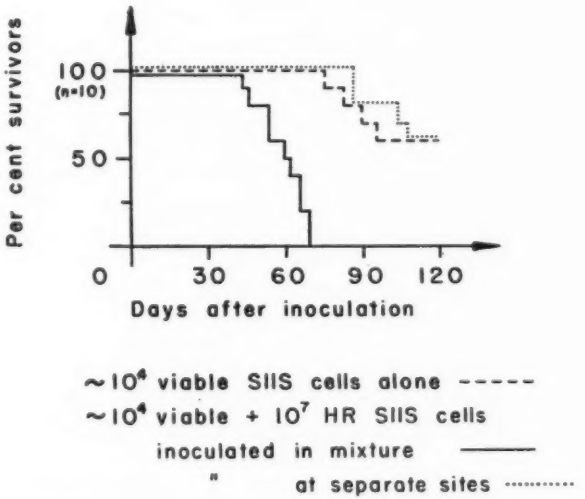
active at the time of inoculation and for some time thereafter in the host, but incapable of continued proliferation under the given environmental conditions.

Efforts were made to maintain the amount of the various admixed materials within roughly comparable proportions. They were all suspended in 3 parts of Ringer's solution to 1 part of tissue. The final inoculum in each case contained 0.1 ml. of this material.

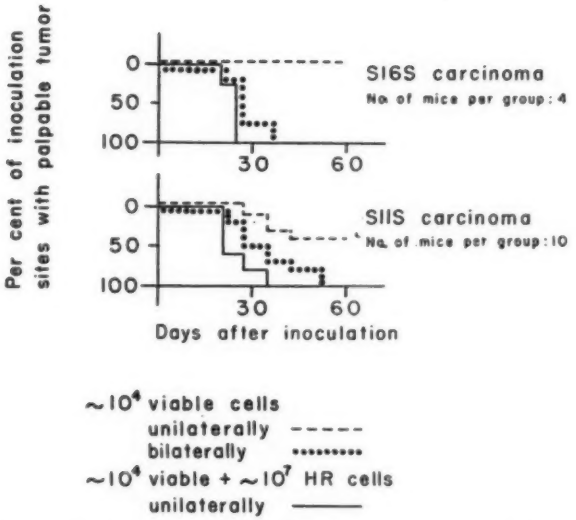
The results are illustrated in text-figure 4. It appears that both isologous liver cells or regressing incompatible tumor cells have stimulated the development of the viable inoculum. The latter result is in agreement with previous findings of Klein and Klein (*15*).

Inoculations at Separate Sites

If the enhancing effect of HR cells is due to some systemic mechanism, mediated through the host, it could be expected that the implantation of HR and viable cells, at separate anatomical sites, would produce an effect essentially similar to their inoculation in mixture. This was not found to be the case. One of three representative experiments is illustrated in text-figure 5. The stimulating effect was completely absent when HR cells were inoculated into the left flank simultaneously with the implantation of viable cells on the opposite side. On the other hand, a certain mutual stimulation could be observed when two *viable* inocula were deposited simultaneously on both sides (text-fig. 6). This is in agreement with the results of Migliarese and Bly, who observed a mutual



TEXT-FIGURE 5.—Survival of (A × A.SW)F_1 hybrid mice inoculated subcutaneously with 10^4 viable cells of the S11S carcinoma of strain A origin administered either alone or together with 10^7 HR cells of the same kind. The HR cells were either admixed to the viable fraction or inoculated at a distant site.



TEXT-FIGURE 6.—Development of palpable tumors in (A × A.SW)F_1 hybrid mice inoculated subcutaneously with 10^4 viable cells of the S11S or S16S carcinomas of strain A origin, respectively, either unilaterally or bilaterally. For comparison, the latency period of tumors originating from a mixture of the same number of viable cells and 10^7 HR cells is also shown.

stimulation with multiple transplants of the Walker carcinoma during the early stages of growth (*16*).

Effect of Local Inflammatory Response

The possible role of a local inflammatory response was tentatively approached by two different procedures. In a series of four experiments, washed and heat-sterilized quartz sand (particles between 5 to 10 μ) was mixed with the tumor-cell suspensions immediately before inoculation. Volumes of 0.2 ml. were inoculated, containing between 0.15 to 1.0 mg. quartz. Two spontaneous carcinomas of a DBA and an A female mouse designated S11D and S17S, respectively, were inoculated into (A × DBA)F_1 hybrids. In two experiments the ELD/Sto and Ehrlich/Sto ascites carcinomas were also tested, using F_2 hybrids of the A and C3H strains for the inoculation of these "nonspecific" tumors. No enhancing effect could be observed in any of these experiments. A certain inhibition was suggested by the decreased frequency of progressively growing tumors (table 3).

A number of other experiments were made on the possible effect of a local allergic inflammatory response at the site of inoculation provoked according to the procedure of Molomut *et al.* (*17*). In one experiment, 15 (A × DBA)F_1 hybrids were sensitized by intraperitoneal injections of normal horse serum in 0.5 ml. doses, administered at 3-day intervals during

TABLE 3.—*Effect of admixed quartz on the growth of various tumor inocula**

Number of cells	Ehrlich/Sto		ELD/Sto		Spontaneous carcinoma of the DBA strain (S11D)		Spontaneous carcinoma of the A strain (S17S)	
	quartz		quartz		quartz		quartz	
	+	−	+	−	+	−	+	−
10^5	3/6	6/6	1/3	3/3				
10^4			0/8	7/8			3/5	4/5
10^3			0/8	5/8	2/8	4/4	1/4	10/10
10^2	1/6	4/6	0/8	5/8	1/8	2/4		
10	0/6	2/6						

*The results are given as the number of animals killed by progressively growing tumors/total number inoculated.

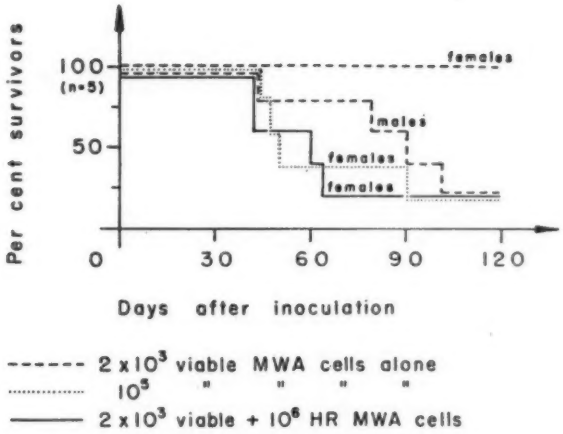
3 weeks. Maximum sensitization can be expected to occur 2 weeks after the last injection, according to Cameron (*18*).

At this point the animals received 0.2 ml. of a tumor-cell suspension subcutaneously, containing about 10^4 viable cells. The cells were derived from a spontaneous mammary carcinoma of an A mouse (designated S19S). Ringer's solution mixed with horse serum in a proportion of 1:5 was used as the suspending medium. A control group of 15 nonsensitized mice received the same inoculum but without previous sensitization. No major difference was found between the two groups. Similar results were obtained in two repeat experiments using 10^3 and 10^2 cells, respectively, derived from the MWA tumor and with $(A \times A.SW)F_1$ hybrid hosts. These findings are in agreement with the results of Molomut *et al.* (*17*).

Experiments with a Partially Dependent Tumor

The MWA tumor originated in a male mouse of the A.SW strain after the intramuscular injection of 1 mg. methylcholanthrene into the right thigh. Histologically it was a polymorphous spindle-cell sarcoma with a high frequency of mitosis.

A small number of cells (about 10^3), when inoculated subcutaneously into 3-month-old A.SW mice of both sexes, grew well in males but very few takes were obtained in females (text-figs. 7 and 8). With larger inocula (about 10^5 viable cells) good growth was obtained also in females (text-fig. 7). When 3-month-old males and females were castrated and challenged 3 weeks later by inoculating 1.5×10^3 viable MWA cells, the results shown in text-figure 8 were obtained. Normal untreated males and females were used as simultaneous controls. Tumors developed most rapidly in castrated females, which was also reflected by the short survival time in this group. Normal and castrated males had a more prolonged survival time, similar for both groups. In normal females tumor growth was considerably inhibited. This inhibition could be counteracted by the admixture of 10^6 to 10^7 HR cells (text-figs. 7 and 8).



TEXT-FIGURE 7.—Survival of male A.SW mice inoculated subcutaneously with 2×10^3 viable MWA cells and of 3 groups of females, inoculated with 2×10^3 or 10^5 viable MWA cells alone or with a mixture of 2×10^3 viable and 10^6 HR cells.

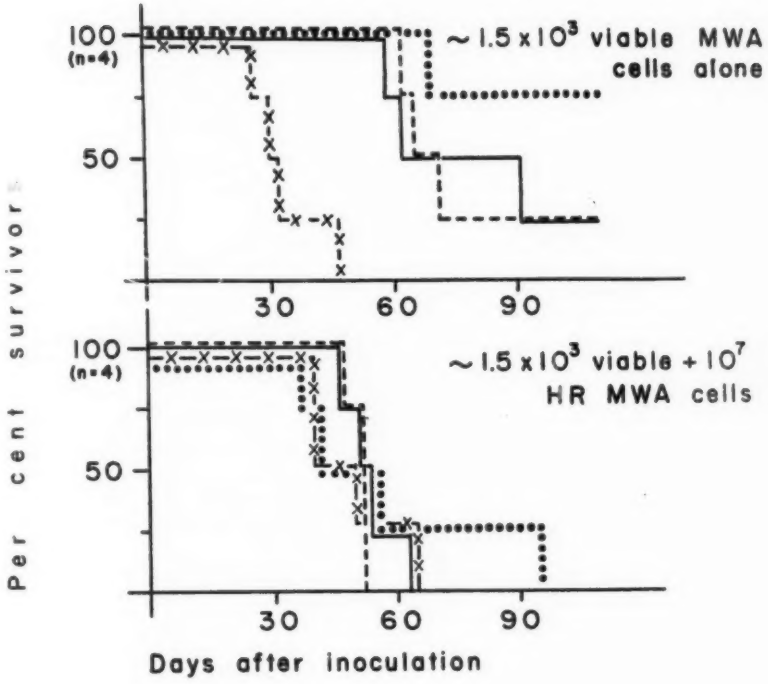
Preirradiated Implantation Sites

Since all experiments described in the previous paragraphs made use of tumor implants growing in the host environment of normal, unirradiated tissues, it was decided to perform similar experiments in animals where the tumor-bed had been irradiated prior to inoculation. On *á priori* grounds, it was uncertain whether the intense stimulating effect of the HR cells would develop under such circumstances. It was also decided to perform additional experiments to obtain some information about the chances of a small number of viable tumor cells surviving within tumors that have received a heavy dose of irradiation *in situ*.

A number of spontaneous tumors from different C3H females (designated S26C, S27C, and S28C, respectively) were used in three different experiments (expts. A, B, and C, of text-fig. 9). The viable cells of the tumors were inoculated in volumes of 0.05 ml., either alone or in mixture with HR cells (treated with 15,000 r), into the preirradiated or untreated hind legs of male C3H or (C3H $\times$ DBA)F_1 hybrid mice according to the schedule shown in text-figure 9. These inoculations were performed by using a 26-gauge needle introduced in proximodistal direction. The results show that tumor proliferation was considerably inhibited in the preirradiated sites. This inhibition is manifested with both viable cells alone and mixtures of viable and HR cells.

The development of the tumors in the bilaterally inoculated mice in experiment C was similar to the results obtained with animals injected on one side only.

In another series of experiments, carcinoma S19S of strain A origin, carcinomas S12D, S13D, and S14D of DBA origin, and sarcoma MNSB of (A $\times$ A.SW)F_1 origin were each inoculated into 40 compatible hosts.

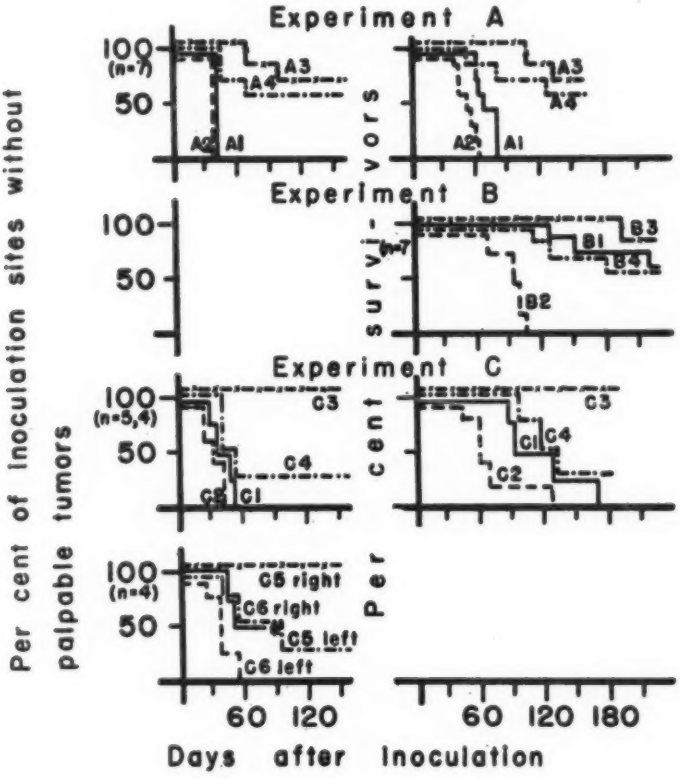


——— Males
– – – – Castrated males
••••••• Females
-x-x- Castrated females

TEXT-FIGURE 8.—Survival of normal and castrated A.SW males and females after the subcutaneous inoculation of 1.5×10^3 viable MWA cells alone or in mixture with 10^7 HR cells of the same tumor.

After 3 to 4 weeks, all mice carrying well-circumscribed tumors of about 1 cm. in diameter were selected. Tumors that showed attachment to the underlying bone or had any signs of ulceration were rejected. From 10 to 20 mice were usually found to carry suitable tumors in each group. The tumors were locally irradiated with doses of 6600 or 7700 r, while the body of the animals was shielded. Diluted cell suspensions prepared from another untreated sample of the same tumor were injected through a medium-point, 26-gauge needle, in amounts of 0.05 ml. containing about 10^3 viable cells, into the center of the irradiated tumors 2 hours after treatment (group RV). Irradiated tumors of a control group (group RO) were injected with 0.05 ml. of Ringer's solution in the same way. Another control group (group OV) consisting of untreated animals of the same strain received the same inoculum subcutaneously into the right leg.

The results of five different experiments (A–E) are shown in text-figure 10. The effect of the high radiation dose on the established tumors varied in the different experiments from complete inhibition in experiment B to various degrees of partial inhibition. All animals that received viable implants into their preirradiated tumors died with progressively growing neoplasms. With the exception of experiment B, they died



TEXT-FIGURE 9.—Inoculation schedule and results of three experiments designed to compare the effect of implanting viable tumor cells alone or in mixture with HR cells into preirradiated or untreated normal tissues, respectively. Mammary carcinomas S26C, S27C, and S28C of C3H origin were inoculated into the hind legs of C3H or (C3H × DBA)F_1 mice. All the animals of group C–6 died from tumors developing at the unirradiated site within 80 days after inoculation.

earlier than the corresponding control group of normal mice inoculated with the same dose of viable cells at an unirradiated site.

Studies with Immunogenetically Incompatible Systems

The effect of HR cells was first studied, by the author, on heterozygous mice bearing intraperitoneally inoculated Ehrlich ascites carcinomas. Both stimulation and inhibition could be found in this system depending upon the quantitative proportions of HR and viable cells. This is in contrast to the exclusively stimulating effect found with genetically compatible host-tumor systems. Because of this difference, and due to the widespread use of host-tumor systems lacking immunogenetical identity for radiobiological and chemotherapeutical experiments, it was decided to collect some more information on the influence of HR cells on viable cells growing in various foreign genotypes. Hosts were selected which would support the progressive growth of particular foreign tumors, at least in a certain proportion, but would nevertheless be immunogenetically competent to react against them.

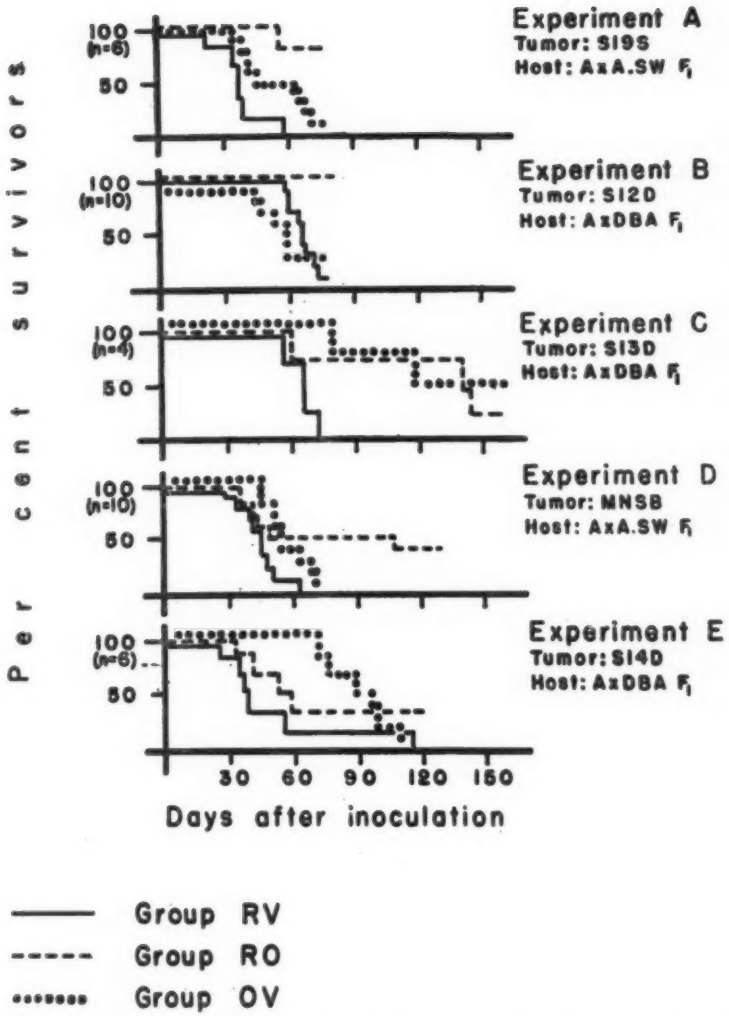
For these experiments the following tumors were used:

1) The Ehrlich/Sto ascites tumor that originated in a mouse of unknown genetic composition and has been propagated in heterozygous animals for more than 60 years (*11*).

2) A variant line (MSWBY) of the methylcholanthrene-induced sarcoma MSWB. The immunogenetical behavior of this tumor and some of its variants has been studied previously in detail (*6*). It has been induced in an F_1 hybrid of the isogenic-resistant lines A and A.SW. Variant lines capable of growing in the A.SW but not in the A parental line have been produced by selection—MSWBY is one of them. This particular

TEXT-FIGURE 9.—Continued

Experiment	Groups	Number of mice	Tumor designation	Treatment of right leg			Treatment of left leg	
				Preirradiation (*r*)	Number of:		Number of:	
					Viable cells	Admixed HR cells	Viable cells	Admixed HR cells
A	1	7	S26C	1200	10^3	10^6	—	—
	2	7	"	—	10^3	10^6	—	—
	3	7	"	1200	10^3	—	—	—
	4	7	"	—	10^3	—	—	—
B	1	7	S27C	1500	10^3	10^7	—	—
	2	7	"	—	10^3	10^7	—	—
	3	7	"	1500	10^3	—	—	—
	4	7	"	—	10^3	—	—	—
C	1	4	S28C	3000	2×10^3	10^6	—	—
	2	5	"	—	2×10^3	10^6	—	—
	3	4	"	3000	2×10^3	—	—	—
	4	4	"	—	2×10^3	—	—	—
	5	4	"	3000	2×10^3	—	2×10^3	—
	6	4	"	3000	2×10^3	10^6	2×10^3	10^6



TEXT-FIGURE 10.—Survival of mice after the inoculation of viable tumor cells (group RV) or of Ringer's solution (group RO) into the center of established preirradiated tumors of the same kinds. The survival of previously untreated mice of the same strain, inoculated with the same number of viable cells subcutaneously, is also indicated (group OV); 7700 r was used for pretreatment of groups RV and RO in experiments A and B, 6600 r in C, D, and E.

variant is also capable of progressive growth in a certain proportion of the A.BY isogenic-resistant line. The growth in A.SW seems to be due to the loss of the isoantigenic components determined by *H-2*[4] and the variant grows progressively, even in animals hyperimmunized with the original line of the tumor. Growth in A.BY seems to be of a different

nature since it does not happen in immunized animals, and in untreated mice it seldom reaches 100 percent.

3) The MSWC sarcoma has been induced by methylcholanthrene in an (A × A.SW)F_1 hybrid mouse. Since its earliest history this tumor has grown in a large percentage of both parental strains and also in foreign genotypes (*6*). It was not possible to isolate clear-cut variants with selective compatibility from this tumor.

The results are shown in table 4. It appears that HR cells may result in either stimulation or inhibition or, alternatively, it may be of no detectable importance, depending on the host-tumor combinations and the cell doses used. This is at variance with the immunogenetically compatible host-tumor systems in which stimulation regularly occurred when HR cells in mixture with viable cells were inoculated, and tumor development was not affected when HR cells were transplanted at a different site. It is plausible to assume that the inhibitory effect in foreign hosts is due to a complicating homograft reaction. If this assumption is correct, it could be expected that the mice in which tumor growth has been inhibited would be resistant to subsequent challenges with large viable inocula from the same tumor. This was borne out by experiments in which a second challenge was given: A large number of viable tumor cells [about 10^7 cells of Ehrlich ascites tumor (*1*) and 0.2 ml. of a 1:3 diluted suspension of tumor tissue-pulp of the MSWC and MSWBY tumors] were implanted in animals that remained negative after the previous inoculation of viable cells alone or a mixture of HR and viable cells of the same kind. The results are summarized in table 5. It appears that while 11 out of 12 (A × A.SW)F_1 mice succumbed to a second inoculation of the genetically compatible MSWC tumor, 54 of 55 genetically incompatible mice survived the second challenge with the MSWC, MSWBY, or the Ehrlich tumor, respectively.

These experiments seem to suggest that, in genetically foreign mice, the enhancing or inhibiting effect observed is the final result of two competing influences: a stimulating effect like what occurs in genetically compatible systems, and an immunizing effect due to the isoantigenic differences between the tumor cells and their host.

Discussion

Tumors irradiated with sublethal X-ray doses can be schematically considered as containing two kinds of tumor cells, differing in their prospective reproductive potentialities. One fraction is composed of cells that have been damaged irreversibly and lost their capacity for continued reproduction, either immediately or after a number of divisions (*19*). Another part of the cell population is composed of cells undamaged or damaged in a reversible way but still capable of multiplying serially without restraint. The relative proportion of these two fractions is probably related to the dose of irradiation, the inherent radiosensitivity of the cells, and to various environmental conditions before, during, and after treatment. Little is known about the influence that the lethally

TABLE 4.—*Incidence of progressively growing tumors after inoculation of tumor-cell suspensions into genetically foreign hosts. All tumors were known to grow progressively in a certain proportion of the foreign genotypes used. Suspensions of viable cells were transplanted alone, in mixture with HR cells, or simultaneously with the inoculation of HR cells at a different anatomical site*

Experiment	Tumor line		Conditions of inoculation			Observation period (*days*)	Proportion of mice with progressively grown tumors*			
	Designation	Mouse strain of origin	Mouse strain inoculated	Number of cells or amount of tissue pulp			Viable cells alone	Viable + HR cells in mixture	Viable + HR cells at separate sites	HR cells alone
				Viable	HR					
1	2	3	4	5	6	7	8	9	10	11
1 †	Ehrlich/Sto	Noninbred	Heterozygous	1.8×10^6	1.6×10^7	12	25/25	25/25		0/14
2 †	Ehrlich/Sto	Noninbred	Heterozygous	10^2	1.8×10^7	90	8/8	1/8		0/8
3 a	MSWBY	(A × A.SW)F_1	(A × A.SW)F_1	10^4	0.025 ml.	90	5/5	4/4	5/5‡	‡
b	"	"	A.SW	"	"	"	5/5	5/5	5/5	
c	"	"	A.BY	"	"	"	0/5	0/4	0/5	
d	"	"	(A × A.SW)F_1	10^3	"	"	5/5	5/5	5/5	
e	"	"	A.SW	"	"	"	5/5	5/5	5/5	
f	"	"	A.BY	"	"	"	3/5	0/5	0/5	
4 a	MSWC	(A × A.SW)F_1	(A × A.SW)F_1	10^3	0.025 ml.	90	0/5	5/5		0/3
b	"	"	(A × DBA)F_1	"	"	"	0/4	4/4		
5 a	MSWC	(A × A.SW)F_1	(A × A.SW)F_1	7×10^3	0.025 ml.	90	5/5	5/5		0/4
b	"	"	(A × DBA)F_1	"	"	"	0/5	0/5		
6	MSWC	(A × A.SW)F_1	A.SW	10^5	0.025 ml.	90	3/6	2/4	0/5‡	‡

*Expressed as the ratio of mice with progressively growing tumors and the total number inoculated.

†These experiments have been published previously in detail (*1*).

‡Mice inoculated with HR cells at a separate anatomical site (col. 10) served simultaneously as controls to check the efficiency of the X-ray treatment in producing complete inactivation of the population.

TABLE 5.—*Incidence of progressively growing tumors in survivors of previous inoculations with viable cells alone or mixtures of viable and HR cells. Previously untreated mice served as controls. Observation period: 60 days.*

Tumor	MSWC—origin in (A × A.SW)F_1								MSWBY—origin in (A × A.SW)F_1							Ehrlich/Sto
Inoculum	0.025 ml. tumor pulp								0.025 ml. tumor pulp							10^7 cells
Genotype of recipient mice	(A × A.SW)F_1				(A × DBA)F_1				A.BY							Hetero-zygous
Previous history of the recipient mice.	Previously untreated controls	Survivors from experiment			Previously untreated controls	Survivors from experiment			Previously untreated controls	Survivors from experiment						Survivors from experiment 2*	
		4 a*		5 a*		4 b*	5 b*			3 c*			3 f*				
		col. 8*	col. 11*	col. 11*		col. 8*	col. 8*	col. 9*		col. 8*	col. 9*	col. 10*	col. 8*	col. 9*	col. 10*	col. 9*	col. 11*
Tumor incidence†	6/6	4/5	3/3	4/4	4/6	0/4	1/5	0/5	11/13	0/5	0/4	0/5	0/2	0/5	0/5	0/7	0/8

*The experiment and column designations refer to table 4.

†Expressed as the ratio of mice with progressively growing tumors and the total number inoculated.

damaged population may exert on the continued proliferation of the surviving fraction, either directly or through the host organism. It is conceivable that the dead or dying cells may stimulate the proliferation of the survivors in a variety of ways, though it is equally possible that they inhibit the survivors, either by some toxic products or as a result of the intense inflammatory reaction induced locally in the host.

The problem was approached by model experiments in which irreversibly damaged tumor cells were mixed with viable or reversibly damaged tumor cells of the same kind in various proportions. In genetically compatible tumor-host systems, cells irreversibly damaged by heavy doses of radiation (HR cells) were previously found to exert a profound enhancing influence on the growth of the admixed viable cell fraction (*2*). This was manifested by an earlier and higher incidence of progressively growing tumors and a decreased survival time of the tumor-bearing animals. In the present investigation, this finding was confirmed and extended to an additional variety of tumors. Efforts have also been made to obtain some information with regard to the mechanisms involved.

In a general way, one may conceive of two major types of mechanism involving (*a*) direct interaction between tumor cells or (*b*) indirect effects operating through the host. These will be discussed briefly in the following:

(*a*) HR cells do not necessarily die soon after irradiation; they may survive for certain periods of time, and it has been shown, although on other systems and with lower X-ray doses than used here, that irreversibly X-ray-damaged cells may indeed enlarge, synthesize proteins and nucleic acids, and perform active metabolic functions (*19*, *20*). Their final disintegration usually ensues in connection with more or less unsuccessful attempts to divide. Before this happens, HR cells might conceivably stimulate a small viable cell fraction randomly intermixed within them by providing various kinds of useful metabolic products, cell organelles, or specific "tissue growth hormones." It is also possible, however, that the viable cells reactivate some HR cells and protect them from death by providing them with materials capable of re-establishing the potentiality for continued proliferation. The occurrence of such effects has been discussed by Delaporte, Latarjet, and Devik (*21–23*). Reactivation of HR cells might lead to enhanced tumor development as a result of the increased initial size of the reproductive population.

(*b*) An indirect stimulation mediated by the host could be due to some general systemic effect or to the local inflammatory response provoked by products of the HR cells. Enhanced vascularization of the viable graft could also contribute to it.

The experiments described in the present paper tend to indicate that metabolically active cells are required for the stimulating effect observed. Thus, in addition to HR cells, genetically incompatible (homologous) tumor cells or genetically compatible (isologous) liver cells were also found to be capable of stimulating the development of admixed viable

tumor cells. Since genetically incompatible tumor cells survive and grow before the ensuing homograft reaction destroys them (a matter of 1 or several weeks) and isologous liver cells may also survive for a certain period of time, these cells share the attribute of initial metabolic activity with HR cells. In contrast, there was no enhancement in the presence of cells killed by prolonged incubation or by heat; on the contrary, a certain inhibition was found.

Before considering possible host-mediated mechanisms it should be pointed out that no stimulatory effect was obtained when viable and HR cells were inoculated separately at different anatomical sites. This would rule out a systemic effect on the host, at least in its pure form. It also suggests that the stimulation by the HR cells is not identical with the phenomenon of immunological enhancement, studied extensively by Casey, Snell, Kaliss, and others (*24–26*). Immunological enhancement deals with genetically incompatible host-tumor systems. It differs from the present phenomenon which applies primarily to isologous and genetically compatible mouse-tumor combinations; also with regard to the time relationships (pretreatment *versus* simultaneous administration) and the nature of the preparations involved (lyophilized *versus* metabolically active tissue). Similar differences in the nature of the stimulating material, time of treatment, and site of administration clearly distinguish the presently described phenomenon from the accelerating effects described by Shear *et al.* and by Miroff *et al.* (*27, 28*). The former authors have used pretreatment with saline extracts of tumor tissue 9 days before inoculation, while the latter group has dealt with heat-denatured tumor tissue, administered 31 days previous to implantation. Both groups used isologous host-tumor systems. The administration site of the accelerant did not coincide with the site of the subsequent implantation. Our phenomenon of stimulation may, on the other hand, be related to the stimulation described by Schneyer (*29*), which occurs upon the simultaneous implantation of tumor and embryo cells.

The lack of activity at distant sites shows, furthermore, that the findings on the present system also differ from the tissue-specific growth stimulation observed after the injection of tissue homogenates into various mammals (*30*). On the other hand, the mutual enhancement of tumor development in bilaterally inoculated *viable* grafts may be analogous to the findings of Teir *et al.* (*30*), though the specificity of our phenomenon remains to be established. Analogous results were reported by Migliarese (*16*).

The question arises whether *local* host-mediated mechanisms could be responsible for the stimulating action of HR cells. Enhanced vascularization of the viable graft is not a very probable explanation, at least in its pure form, since it was found (*1*) that HR cells were quite capable of stimulating ascites tumors when growing in the form of freely suspended cells in the peritoneal fluid with no stroma and no vessels. The stimulation was most intense at an early stage of ascites-tumor development before the appearance of more extensive solid tumor implants.

Another possibility would be that the local inflammatory reaction provoked by the HR cells (*1*, *20*) would promote the growth of viable tumor cells. In this connection it may be pointed out that the present experiments demonstrate how little damage such a host response can inflict upon a small number of genetically compatible and viable tumor cells randomly distributed in the reaction area; in fact, the opposite effect might be true. To investigate the latter possibility, some experiments were carried out to see whether local inflammation alone would promote tumor growth in our particular system. Reactions provoked by the injection of quartz, allergene or heat-killed cells did not result in any detectable enhancing effect. In some cases, an inhibiting effect could be observed. This would suggest that inflammation *per se* is not necessarily capable of stimulation. This is in conformity with the conclusions of Molomut *et al.* (*17*) and of Chambers and Grand (*31*). The possibility remains, however, that the particular inflammatory reaction provoked by HR cells is different in nature from the responses produced in these model experiments and the possible significance of the local host response cannot be critically dismissed on the basis of the present results. Other types of local inflammatory reactions have been reported to stimulate tumor growth under various experimental conditions (*32*, *33*).

No evidence is available with regard to the great variety of possible mechanisms of a cell-to-cell stimulation which may be due to various substances contributed by HR cells. Alternatively, they may act by absorbing toxic products. Detailed speculations would be hardly profitable at the present stage. One piece of potentially valuable information may be mentioned, nevertheless. The stimulation of MWA sarcoma cells by HR cells, in female mice, could possibly indicate a neutralization of some inhibiting factor present in the females. This is not the only possibility, however, since the stimulating effect observed might equally well be due to the combined end result of growth inhibition in the females and the counteracting stimulating influence of HR cells.

The sex dependence of MWA was only apparent when small inocula were used such as 10^3 cells. Large inocula grew well in both females and males. A similar finding has been reported (*34*) on an estrogen-dependent mammary carcinoma of the A strain. These findings may suggest that a large number of cells are capable of building up a sufficiently high concentration of growth-stimulating substances. These substances, which could be contributed by HR cells as well, would be capable of obviating the dependence on exogenous hormones in a functional sense. If true, this would imply that progression of hormone-dependent tumors to states of unresponsiveness might conceivably occur as a result of the fact that the cells gradually acquire the ability, either by way of adaptation or by selection of variant types arising *de novo*, to produce and utilize endogenous substances, which are capable of acting as substitutes for the exogenous stimuli previously needed. In a small population such cell products may become too diluted and fall below an effective level and the cells once more become dependent on the previously needed exogenous stimulus.

The stimulating effect of HR cells was most apparent when the proportion of viable cells in the inoculated mixture was comparatively low. This is probably the reason why Henshaw (*35*) failed to detect any difference between the survival of mice inoculated with viable cells alone and with a mixture of viable and HR cells of sarcoma 37, respectively. The proportion of viable cells in his experiments was never less than 1:4, and heavy inoculum doses were used. Only minor stimulating effects could be expected under such conditions and they may be discovered with the help of quantitative growth curves as shown with the Ehrlich ascites tumor (*1*) but may not be detected by coarser methods of observation.

Stimulation of cellular proliferation by the products of adjacent cells of the same kind has occurred in different experimental systems. Thus, the growth-lag of bacteria (*36*) and of protozoa (*37*) was found to decrease with increasing inocula. It was shown by Sanford *et al.* (*38*) that the growth of single tissue cells *in vitro* can be promoted by the use of "conditioned" media taken from growing cultures. Stimulation of the growth of single HeLa tumor cells by products of homologous cells damaged lethally by X rays was demonstrated *in vitro* by Puck (*39*). Loofbourow has found that growth-promoting factors were released into the intercellular fluid when yeast cells were damaged by such widely different agents as ultraviolet radiation or mechanical or chemical injury (*40*). Recently, Powell (*41*) has found that tumor cells mutually protect each other's viability *in vitro* and that the frequency of cell divisions is stimulated in dense, as contrasted to dilute, cell populations. It has been demonstrated previously that the latency period in the growth curve of ascites tumors *in vivo* is inversely related to the inoculum size (*11*). An interdependence of certain kinds of ascites-tumor cells is indicated by the finding that a critical inoculum dose is necessary for their growth in the ascites form, while subthreshold inocula grow as solid tumor (*42*). During the progressive evolution of such cells in continued subpassage, they may nevertheless become independent of such interdependence; ascites tumors can be obtained by the inoculation of single isolated tumor cells after this has occurred (*34*).

What bearing, if any, do the experiments described in this paper have with regard to experimental and clinical approaches toward cancer therapy? There seem to be three main implications:

1) Experimental studies on tumor therapy, whether by X rays or chemical means, should be conducted preferably on isologous, as contrasted to homologous, host-tumor systems. A certain treatment that will damage only one part of the cell population may lead to complete regression of progressively growing but immunogenetically foreign tumor homotransplants after receiving help from homograft reaction of the host. This can result in fallacious interpretations regarding the efficiency of the treatment. Similar conclusions have been reached by Cohen and Cohen (*43*). They consider the radiosensitivity of transplanted tumors, in genetically unidentical hosts, as a quantitative measure of subliminal host resistance resulting from immunogenetical differences in the host-

tumor relationship. This may be one of the reasons for the frequent findings that transplanted tumors can be cured more easily than their spontaneous counterparts. Similar effects must also be considered when interpreting certain findings of Sugiura (*44*), Ahlström *et al.* (*45*), Voutilainen (*46*), and others. These authors have found that the irradiation of a homotransplantable tumor in a genetically foreign host may lead to an inhibition of the growth of another nonirradiated tumor of the same kind, growing at a different anatomical site in the same host.

2) Because of the complex nature of the interactions between viable and dead cells, it is hardly possible to obtain information about the number of tumor cells surviving a certain treatment by quantitatively comparing the growth curve of the treated tumors with a series of calibration curves obtained with various doses of viable untreated cells of the same tumor.

3) Destruction of *all* tumor cells would seem to be the necessary goal of tumor therapy and it is doubtful whether partial destruction of the tumor-cell population may ever be sufficient. The view is often expressed that partial damage may lead to satisfactory results, due to the irradiation of the tumor stroma, with consecutive necrobiosis of those tumor cells that have survived treatment, and also because of hypothetical defense mechanisms of the host. Attempts were made in the present investigation to assess the possible role of these factors. It was found that the enchancing effect of the HR cells becomes apparent not only when untreated viable cells are used as targets but also in situations where the target cells have been reversibly damaged by X rays. A similar stimulating effect may conceivably occur in irradiated tumors in which one part of the population is completely inactivated, while another part has only suffered reversible damage. The clinical situation is different from these model systems, however; one of the main differences lies in the fact that the stroma is irradiated together with the tumor cells. We have therefore performed some experiments in which the site of inoculation has been preirradiated. In agreement with the findings of Vermund *et al.* (*47*), a certain inhibition of tumor growth was found to result from preirradiation of the normal tumor-bed; this effect was partially counteracted by the opposite stimulating influence of HR cells. Inoculation of a small number of viable cells into the center of established and heavily preirradiated tumors of the same kind has shown that the viable cells can survive and grow regularly in spite of the damaged environment, comprising in this case both tumor cells and stroma. Thus, while damage to the stroma and the tumor-bed may have an inhibiting influence on the growth of the survivors, this inhibition can be counteracted, at least partially, by the enchancing effect exerted by irreversibly damaged tumor cells.

The present experiments have failed to provide any evidence that would indicate the existence of efficient host-defense mechanisms in genetically compatible host-tumor systems. This becomes particularly apparent when considering the inability of the host to destroy a small number of viable cells that constitute a minute fraction of a large and

irreversibly damaged tumor-cell population in a normal, untreated animal. However, these conclusions apply only to the investigations described in this paper. On the other hand, it has been clearly shown that defense reactions do occur when there is a genetic difference between tumor cells and their hosts; these reactions seem to be of the nature of the classical homograft reaction.

Summary

The influence of a large number of dead or irreversibly damaged tumor cells upon the subsequent proliferation of a small, randomly admixed viable cell fraction was studied in a series of transplantation experiments in highly inbred mouse strains.

Cell suspensions prepared from a variety of mammary carcinomas, methylcholanthrene-induced sarcomas, and an ascites lymphoma were inactivated by lethal doses of X rays *in vitro*. Subsequently, they were mixed with viable or reversibly damaged cells of the same kind in various proportions. These mixtures were found to grow more rapidly in compatible hosts than the same number of viable cells alone. The enhancement was manifested by an earlier appearance and a higher incidence of progressively growing tumors and a decreased survival time of the tumor-bearing animals. It was most apparent when the number of viable cells was relatively small. No enhancement was found when the lethally damaged cells were deposited at a site anatomically remote from the viable cells.

Implantation of a small, viable inoculum into preirradiated normal tissues resulted in a certain growth inhibition. This inhibition could be partially counteracted by large numbers of lethally X-ray-damaged tumor cells in the inoculum. Implantation of small numbers of viable tumor cells into the center of established tumors of the same kind, which were heavily preirradiated *in situ*, demonstrated that viable cells can survive and grow under such conditions.

Isologous liver cells and genetically incompatible tumor cells were also found to exert a stimulating influence on the growth of an admixed, small, viable, and compatible tumor-cell fraction. Homologous tumor cells killed by heat or by prolonged incubation were inactive.

Local inflammatory reactions at the implantation site, provoked by quartz pulver or by an allergene, did not result in any detectable enhancing effect; an inhibition could be observed in some cases.

In mice genetically different from the original host of the tumor, lethally X-ray-damaged cells could either stimulate or inhibit the development of a small, viable fraction, depending on the quantitative proportions of the two kinds of cells and the tumor-host combination used. In cases of inhibition, the surviving hosts could be shown to develop resistance to subsequent challenges with large, viable inocula derived from the same tumor. This, and the complete absence of similar observations on genetically compatible tumor-host systems, indicated that the inhibiting effects observed are due to active immunization of the host

against the foreign isoantigens carried by the genetically unrelated tumor cells.

Various conceivable mechanisms were considered in trying to interpret the enhancing effect, and the possible bearing of the results on experimental and clinical approaches toward cancer therapy was discussed.

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Studies of Heterografts in Diffusion Chambers in Mice [1, 2]

GLENN H. ALGIRE,[3] MARY L. BORDERS, *and* VIRGINIA J. EVANS,[4] *Laboratory of Biology, National Cancer Institute,*[5] *Bethesda, Maryland*

In previous work, diffusion-chamber methods were developed (*1*, *2*) which permitted grafts to survive and grow when separated from host tissues by means of porous but cell-impenetrable filters. Homografts survived indefinitely within these chambers and neither initiated immunity in the host (*2*) nor were destroyed if the host had been previously immunized (*1*, *3*). Earlier studies showed that heterografts of mouse-lung rudiments in diffusion chambers survived for at least a week in control rats (*3*) but were destroyed within 2 to 6 days in previously immunized animals.

The relatively long-term studies of heterografts in diffusion chambers, which are reported here, were undertaken to extend these studies to cells of other types and species; to compare heterografts with homografts in their initiation of, and their response to, immunity; and to ascertain the practical limitations of experiments using human cells or cells of animal species in mice for such studies as the mechanism of action of physical or chemical agents. In this paper, observations are reported on the growth of human and of animal cells in diffusion chambers. In a subsequent paper the role of various factors that influence the duration of survival of heterografts in diffusion chambers will be considered.

Materials and Methods

The description given here of diffusion-chamber methods used in these experiments is modified from that described previously (*1*). The specific conditions for the experiments on heterografts are given in detail.

Diffusion-Chamber Methods

Text-figure 1 shows the steps in the construction and assembly of diffusion chambers for intraperitoneal implantation. The filters are sealed to the inner (A) and outer (B) plastic rings. These parts are sterilized with

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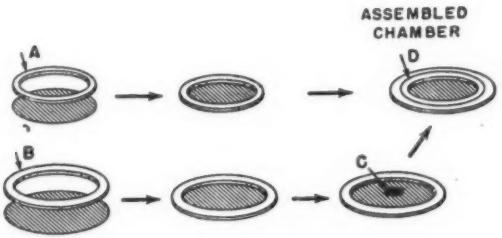
ethylene oxide vapor. A fragment of tissue or a cell suspension (C) is placed upon the filter surface. The surfaces of the filters are approximated as shown (D), and the assembled chamber is then sealed along the juncture of the inner and outer rings. The chambers are placed within the peritoneal cavity and removed later for histologic examination. The details of these procedures will be described. Materials were obtained from the companies mentioned.[6]



TEXT-FIGURE 1.—Construction and assembly of diffusion chamber.

Fabrication of plexiglas rings for diffusion chambers.—The outer rings are 17.5 mm. outside diameter, 14 mm. inside diameter, and 0.75 mm. thick; the inner rings, 13.9 mm. outside diameter, 10.0 mm. inside diameter, and 0.5 mm. thick. The rings are made by means of a punch-press operation using sheets of plexiglas 0.03-inch thick. To minimize loss due to breakage, infra-red radiation is used to preheat the materials. As an alternative procedure, the rings may be made from a solid rod cut from a plexiglas sheet ¾-inch thick. The four-position, tool-post turret attachment of the lathe is used to obtain the desired inside and outside diameters and thickness.

Attachment of filter to plastic rings.—A metal punch is used to cut the filter to fit the above rings. Alternatively, filter material may be obtained on special order to fit rings of the dimensions given above. A 1 percent solution of plexiglas in acetone is used to seal the filter to the ring.[7] The ring is held with forceps while a thin layer of glue is applied to one surface with a cotton applicator. As the wet surface of the ring is applied to the filter, the two materials become adherent and the surface of contact of the filter becomes transparent. Additional glue is used about the edges, if necessary, to complete the seal.

Special large chambers are formed from a plexiglas cylinder, 17 mm. outside diameter, 14 mm. inside diameter, and 5 mm. in height, with a capacity of 0.75 ml. A 1 mm. hole is drilled through the wall of the cylinder for access with a hypodermic needle. Filters are sealed to each end of the cylinder as described.

[6] *Plexiglas II non-UVA formulation:* Sheets—0.03" thick, plus 0.02" or minus .012" may be obtained on special order from Rohm and Haas Co. (minimum order 150 sq. ft. at $1.00 per sq. ft.). Sheets ¾" thick—Gilbert Plastic Supply Co., 21 S. Charles St., Baltimore, Md.

Filter material: Millipore Filter Corp., Watertown 72, Mass., and Carl Schleicher and Schuell Co., Keene, N. H.

[7] In recent experiments, Acryloid B-7, Rohm & Haas Co., Philadelphia, Penna., has been substituted for the plexiglas-acetone solution. The ethylene dichloride solvent of this adhesive is toxic, and its use may result in fatalities if care is not taken to bring about its complete evaporation.

Sterilization procedures.—The filters are exposed to ethylene oxide vapor in a desiccator overnight. Liquid ethylene oxide (0.75 ml. per l. of contained volume) is delivered by using a hypodermic syringe for transfer from storage bottle to desiccator. After sterilization all traces of the gas are removed by aeration. To do this a desiccator with interchangeable tubulature-type outlet is connected to an aspirator filter pump and a water faucet. After exposure to gas overnight, the tubulature outlet is opened, and the water turned on for 1 hour to draw off the gas.

Ethylene oxide is potentially explosive and toxic. Sterilization must be done in a well-aerated room or in a hood. The material is stored before use for at least 2 days after sterilization to permit escape of residual ethylene oxide.

Storage and handling of ethylene oxide.—1) A 100 gm. ampoule of ethylene oxide is placed in a beaker containing ice (under hood). 2) The ampoule is opened (under hood) and the contents are transferred to polyethylene bottles with screw caps. 3) Bottles of polyethylene are placed in a beaker, surrounded by 1 inch of insulating packing, and stored in the freezing compartment of a refrigerator (spark-proof).

Tissue transplantation and assembly of chamber.—In assembling the chamber prior to introduction into the mouse, the filter attached to the outer ring is placed on a drop of tissue-culture medium.[8] The upper surface of the filter is moistened with a drop of the tissue-culture medium and a fragment of tissue is placed in the drop. When a cell suspension is used, 0.01 cc. is placed on the filter. The inner ring with attached filter is then set in place. Care must be taken that the upper surfaces of the plastic rings remain dry. The junction of the inner and outer rings is sealed with a 1 percent solution of plexiglas in acetone (*see* footnote 7), with care to avoid excess. The assembled chambers are kept moist with the tissue-culture medium until they are placed intraperitoneally or subcutaneously into the animal. Approximately 15 minutes is allowed for evaporation of acetone before the chambers are placed within the animal.

A hypodermic syringe is used for the inoculation of cell suspensions into the special large chambers through the access hole on the side wall. The opening is then closed with a tapered plug, and sealed with the plexiglas-acetone solution.

Experimental Procedures

The heterografts used in the following experiments were from strains of HeLa cells and human skin epithelium which had been carried for several years as cell suspensions in continuous culture *in vitro* (*4*, *5*); from human tissue obtained shortly after removal *post mortem* or after biopsy;

[8] The tissue-culture medium consists of 6 cc. balanced saline with tris buffer, 4 cc. horse serum, 0.2 cc. penicillin-streptomycin solution, containing 5 mg. per cc. of each (Microbiological Associates, Bethesda, Md.). A balanced saline with tris contains 0.3 gm. $CaCl_2$, 7.087 gm. NaCl, 0.4 gm. KCl, 2.0 gm. dextrose, which may be omitted, 2.43 gm. tris(hydroxymethyl)aminomethane, .020 gm. phenol red, 16.5 ml. *N* HCl to *p*H 7.25, and water to 1 liter. In the experiments reported here, 4.5 gm. of NaCl were used. The balanced saline with tris and the horse serum are sterilized by filtration through Seitz candles of #03 porosity.

from leukemia cells obtained from peripheral blood of patients; and from the Rous chicken sarcoma.

The cells from tissue culture were obtained from T-60 flask cultures that had been subcultured 7 to 10 days previously. The flask was vigorously shaken to remove cells from the flask floor and to suspend them in the fluid medium. The contents of the flask were centrifuged for 20 minutes at 400 r.p.m., and a portion of the supernatant fluid was removed, so that a final concentration of approximately 200,000 cells per 0.01 cc. was attained when the cells were suspended. The initial inoculum into the diffusion chambers therefore contained about 200,000 cells in 0.01 cc. of fluid.

When solid tissue was used, a small fragment of tissue 0.5 to 1.0 mm. in diameter was placed within the chambers. In studies with peripheral blood from patients with myelogenous leukemia, blood was drawn into a syringe containing heparin. To each 100 cc. of heparinized blood, 15 cc. of a mixture containing 30 mg. of fibrinogen per cc. of human serum was added and mixed thoroughly. The blood was allowed to stand at room temperature in a graduate for approximately ½ hour. By this time the red cells had settled in the graduate leaving a clear fluid at the top which contained white cells and platelets. The white blood cell suspension was placed into HA chambers, using 0.01 cc. inoculum.

In several experiments in which fetal adrenal tissue was used, provision was made for later subcultivation by placing a small disk (3 mm. diameter) of type AA membrane adjacent to the primary explant (fig. 1). At various intervals the chambers were removed and the small disks with attached cells were transferred to second chambers which were implanted as before.

The viability of cells was estimated and graded from 0 (no cell damage) to 6 (complete destruction). Intermediate conditions were denoted by "1," "2," "3," "4," "5," indicating an increased proportion of cells showing evidence of damage. In most cases, cellular degeneration was easily recognized by the presence of cells with pyknotic nuclei, or by disintegration into amorphous cellular debris.

Mice of the C3H/HeN strain (*6*), ranging in age from 3 to 6 months, were used as hosts.

The Millipore membranes used in the following experiments were of types AA, 0.8μ average porosity; HA, 0.45μ; PH,0.3μ; VM, 0.05μ, and VF, 0.001μ (data supplied by the manufacturers).

Results

HeLa Cells in Type HA, VM, and VF Chambers

HeLa cells in HA diffusion chambers placed in normal mice were in good condition and growing rapidly at 7 days (fig. 2 and table 1, A). By 15 days at least one area was found where degenerating cells predominated (figs. 3 and 4). Diffusion chambers removed at 35 to 40 days showed areas in all stages of degeneration in addition to varying proportions of healthy, dividing cells (table 1, A). It is of interest that HeLa cells

were in excellent condition at 21 days in 2 chambers which inadvertently had received a small inoculum.

TABLE 1.—*Growth of HeLa cells in diffusion chambers*

Expt.	Days	Type filter	Average porosity of filters (μ)	Histologic grade: 0	1	2	3	4	5	6
A	7	HA	.45	18*	6	1	1			
	14–15	HA	.45				12	18	14	1
	19	HA	.45					2	1	
	35–40	HA	.45				2	3	1	1
B	15	VM	.050			4	4	1		
	15	HA	.45			3	4	1		
C	15	VF	.001				1	3	1	2
	15	HA	.45			2	3	4		

*Number of animals.

Comparisons were made between HeLa cells in HA, VM, and VF chambers to test the effect of smaller porosity filters on cell survival. These chambers were removed from mice at intervals from 7 to 21 days. After 7 days in VM chambers, the HeLa cells were in good condition, but by 15 or 21 days there was cell destruction of approximately the same degree as in comparable HA chambers (table 1, B and C).

In another experiment (table 1, C) HeLa cells were placed in VF and HA chambers. As seen in table 1, C, cell degeneration after 15 days appeared to be greater in VF than in HA chambers. This suggested that nutritional exchange in the smaller porosity (VF) was less adequate than in the HA chambers.

Skin Epithelium in HA and VM Diffusion Chambers

Cells from a long-term tissue-culture strain of human skin epithelium (*5*) were placed in HA and VM diffusion chambers. The cells appeared healthy at 7 days as shown in figure 5 and table 2. Although a greater

TABLE 2.—*Growth of human skin epithelium in diffusion chambers*

Expt.	Days	Type filter	Average porosity of filters (μ)	Histologic grade: 0	1	2	3	4	5	6
A	7	HA	.45		3*					
	10	HA	.45		1	2	1			
	12	HA	.45			1	1			
	14	HA	.45					2		
B	7	VM	.050		1					
	10	VM	.050		1					
	25	VM	.050		1	1				
	49	VM	.050		1	3	2			

*Number of animals.

degree of cellular degeneration was observed with increased time in the chambers, it is emphasized that even after 49 days, skin cells in VM chambers showed many healthy cells (fig. 6).

Melanomas in HA Diffusion Chambers

HA diffusion chambers containing fragments of biopsy tissue from two human melanomas were put into mice. After 8 days, broad strands of rapidly dividing, mostly nonpigmented cells were found migrating from the original implant of melanoma #1. After 32 days, 1 chamber showed a sparse outgrowth of predominantly pigmented cells in good condition. Five of 6 chambers fixed at intervals up to 47 days contained outgrowths of healthy, dividing cells.

After 7 days, chambers containing melanoma #2 had areas of good cells within the original implant which showed sheetlike organization (fig. 7). The outgrowth from the original implant was often a combination of epithelial sheet and fibroblast-like cells. After 35 days, 1 chamber showed a heavy "fibroblastic" outgrowth with heavily pigmented cells next to the original implant and scattered pigmented cells far from the original implant. Four of 5 chambers fixed at intervals up to 35 days contained outgrowths of healthy cells.

Islet-Cell Tumor

Pieces of human islet-cell tumor tissue were maintained in tissue-culture flasks in a medium of human serum and embryo extract for 1 week before small fragments were placed into type HA, PH, or VM diffusion chambers. After 7 days in mice, the chambers showed a heavy outgrowth of spindle-shaped cells (fig. 8). A general appearance of slow cell degeneration had begun after 17 days and was more extensive after 30 days. However, 15 of 16 chambers fixed at intervals up to 45 days contained some healthy, dividing cells. Three cultures were repeatedly transferred at intervals of 9 to 10 days and were found to be actively growing 2 months later.

Adrenocortical Tumor

Small pieces from the primary tumor and liver metastases of a human adrenocortical tumor were carried for 10 days in tissue culture with antibiotics to clear up infection.[9] Fragments of tissue from both sites were then placed in HA and VM diffusion chambers. The fate of the heterograft was the same for the primary tumor and the metastases in both types of filters. After 11 days, there was usually a good outgrowth of spindle-shaped cells (fig. 9). After 42 and 60 days in the same type chambers, there was the same appearance of slow cell degeneration as with the islet-cell tumor. Of a total of 16 chambers, 13 fixed at intervals up to 73 days contained viable cells, with some mitoses.

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[9] These were subjected to 100 units of streptomycin and 200 units of penicillin in 1 ml. of culture medium made up of human serum and embryo extract.

Human Fetal Ovary and Adrenal Cortex

Explants of fragments from an 8-month-old human fetal adrenal cortex and ovary were made into diffusion chambers. Excellent outgrowth of fibroblast-like cells from ovarian explants was found in 3 chambers fixed at 9, 20, and 30 days, respectively.

Subcultures were made at intervals of 4 to 7 weeks with discs of attached outgrowth of cells from fetal adrenal cortex in one HA-type chamber and from 5 large chambers with HA or VM membranes. The subcultures were fixed after 3 to 4 weeks and found to contain excellent outgrowth of cells from the disc (fig. 10).

Myelogenous Leukemia

Explants were made into chambers of buffy coat from 3 patients with myelogenous leukemia. Numerous healthy cells were found in 17 of 21 chambers fixed before 20 days (fig. 11). After that time, however, the cell population decreased so that only 3 of 14 chambers which were removed 20 to 30 days after implantation contained living cells. Mitoses were infrequent but were found in several chambers removed before 20 days. Eosinophils and immature neutrophils appeared in all chambers up to 20 days.[10]

Rous Sarcoma

Explants of Rous sarcoma were made into VM-type chambers and fixed after 4, 10, 20, and 30 days. Two chambers fixed at 4 days and 2 at 10 days showed abundant outgrowth of fibroblasts, with many dividing cells (fig. 12). At 20 days, although outgrowth was profuse, slight degenerative changes were observed in each of 4 chambers. One chamber fixed at 30 days contained no living cells but considerable cellular debris.

Discussion

Several human tumors have been grown in experimental animals subjected to total X irradiation or to cortisone treatment (*7*, *8*). Survival of heterografts has been attained also in such special sites as the anterior chamber of the eye and the brain of experimental animals (*9*, *10*). Heterografts in untreated experimental animals, however, usually fail to survive or to grow progressively.

Attempts to prolong the survival of heterografts by placing the grafts within collodion bags were reported by Bisceglie (*11*), who found that the Ehrlich mouse carcinoma survived for 12 days under these conditions when placed within the peritoneal cavity of guinea pigs. Eschenbrenner and Francis (*12*) have published a preliminary report of the growth of monkey kidney cells in diffusion chambers in mice.

In the present study, diffusion-chamber methods were used to investigate the fate of heterografts which were separated by porous membranes

[10] We wish to thank Dr. James Stengle, National Cancer Institute, Bethesda, Md., for assistance in identifying cell types.

from contact with host tissues. Advantages of this system are: 1) intermingling of graft and host cells is prevented, thus permitting an accurate assessment of the growth of the cultures; and 2) normal animals may be used rather than those subjected to radiation or cortisone.

Heterografts from several human tissues and the Rous sarcoma survived or grew within diffusion chambers for variable intervals of time. Degenerative changes were inconspicuous before 15 days. From this time on, variable degrees of cellular degeneration were found in all chambers, though many healthy, dividing cells were present up to the termination of experiments—after 60 days.

Several factors should be considered in evaluating these results. No doubt various cell types in diffusion chambers, as in the ascites forms described by Klein (*13*), differ in their ability to use available nutrients. Other factors to be considered are the effects of crowding and the accumulation of waste products.[11] The influence of immune factors on heterografts in diffusion chambers will be discussed in a subsequent paper.

It was interesting to note that the two human cell strains already adapted to rapid tissue-culture proliferation tended to maintain their typical morphological patterns and rapid growth characteristics in diffusion chambers. They also exhibited earlier and more severe degenerative changes than cells obtained directly from surgical specimens which spread out slowly from the original explant. Except for the melanoma and myelogenous leukemia, cell types from surgical specimens could not be identified.

The survival of heterografts in chambers using membranes of an average porosity of 10 millimicrons was not significantly altered.

Diffusion chambers provide a means for short-term studies of the response of human or other heterograft cells to various experimental procedures. Direct and indirect effects of radiation, drugs, or of viruses on cells, for example, can thus be analyzed, and comparisons made with the responses of cells in tissue culture, or as vascularized grafts. It is also possible, if desired, to make subcultures from the original explant, as done *in vitro*.[12]

Summary

Heterografts of various human tissues and of the Rous sarcoma were studied in diffusion chambers for intervals of time up to approximately 50 days.

Two cell strains from tissue culture (HeLa and skin epithelium) grew rapidly without evidence of degenerative changes for approximately 15 days. Varying proportions of healthy and degenerated cells were found thereafter for periods up to 40 days (HeLa) and 49 days (skin epithelium).

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[11] One of us (V. J. E.) reports that under conditions of growth extending beyond the logarithmic phase, some disintegration occurs in massive cell-suspension tissue cultures, apparently accompanied by prolongation of the intermitotic interval.

[12] Acknowledgment is made to Doctors Emil Frei and James Stengle for human myelogenous leukemia; to Dr. Ray Bryan for Rous sarcoma; to Dr. Clyde Brindley for human melanoma tissue; to Dr. Roy Hertz for the islet-cell and adrenocortical tumors, National Cancer Institute, Bethesda, Md.; and to Dr. James Young, National Naval Medical Center, Bethesda, Md., for the human fetal tissues.

Similar results were obtained with explants from surgical specimens of melanoma, islet-cell tumor, adrenocortical tumors, fetal ovary and adrenal cortex, and the Rous sarcoma.

Buffy coat from peripheral blood of three patients with myelogenous leukemia survived for only 20 days. Eosinophils and immature neutrophils were present, but mitoses were infrequent.

It appears that short-term experimental studies of human or other heterograft cells in diffusion chambers in normal, untreated mice are entirely practicable under these experimental conditions.

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PLATE 114

FIGURE 1.—Outgrowth from human adrenal tumor after 11 days in VM chamber. *Arrow*—original explant (*see* text). × 30

FIGURE 2.—HeLa cells after 7 days in HA chamber showing healthy condition of cells, with cell division. × 1,000

FIGURE 3.—HeLa cells after 15 days in HA chamber showing healthy cells. × 500

FIGURE 4.—Same chamber as figure 3 showing area of cellular degeneration. × 500

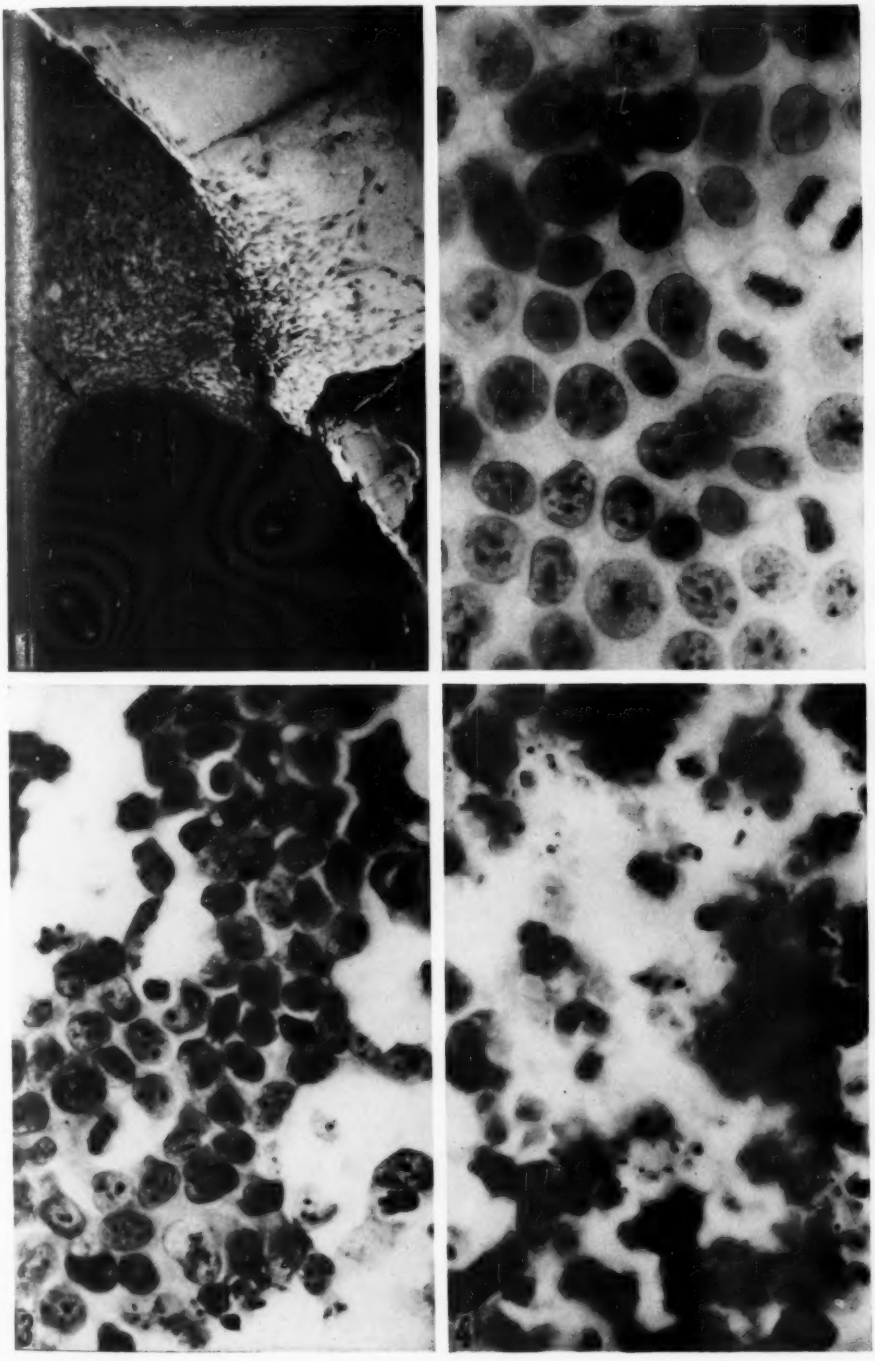

PLATE 115

FIGURE 5.—Human skin epithelium after 7 days in VM chamber showing healthy condition of cells, with cell division. × 800

FIGURE 6.—Human skin epithelium after 49 days in VM chamber showing many healthy, dividing cells together with some degenerating cells. × 500

FIGURE 7.—Human melanoma after 7 days in HA chamber. × 800

FIGURE 8.—Human islet-cell tumor after 21 days in HA chamber showing spindle-shaped cells (subculture). × 30

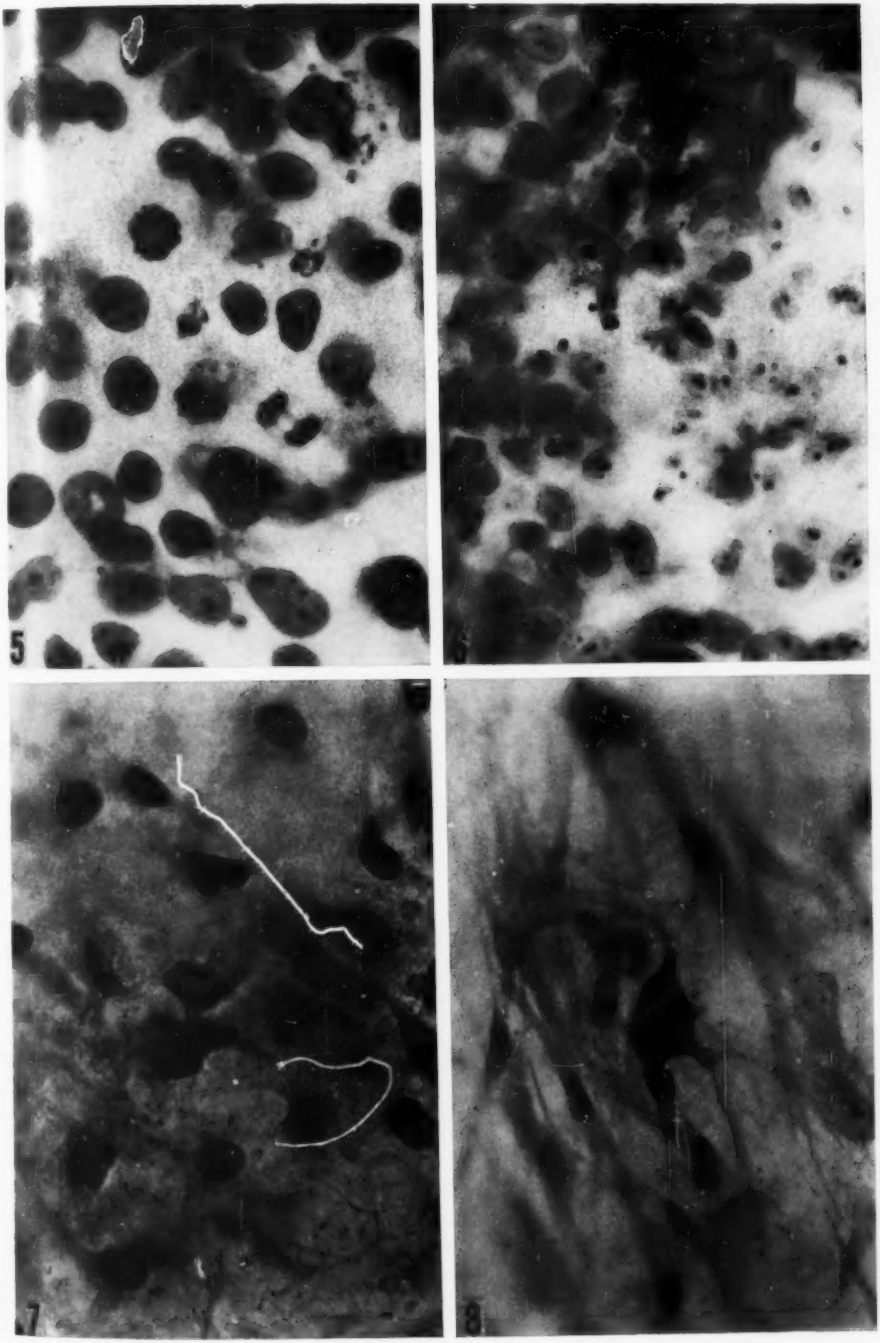
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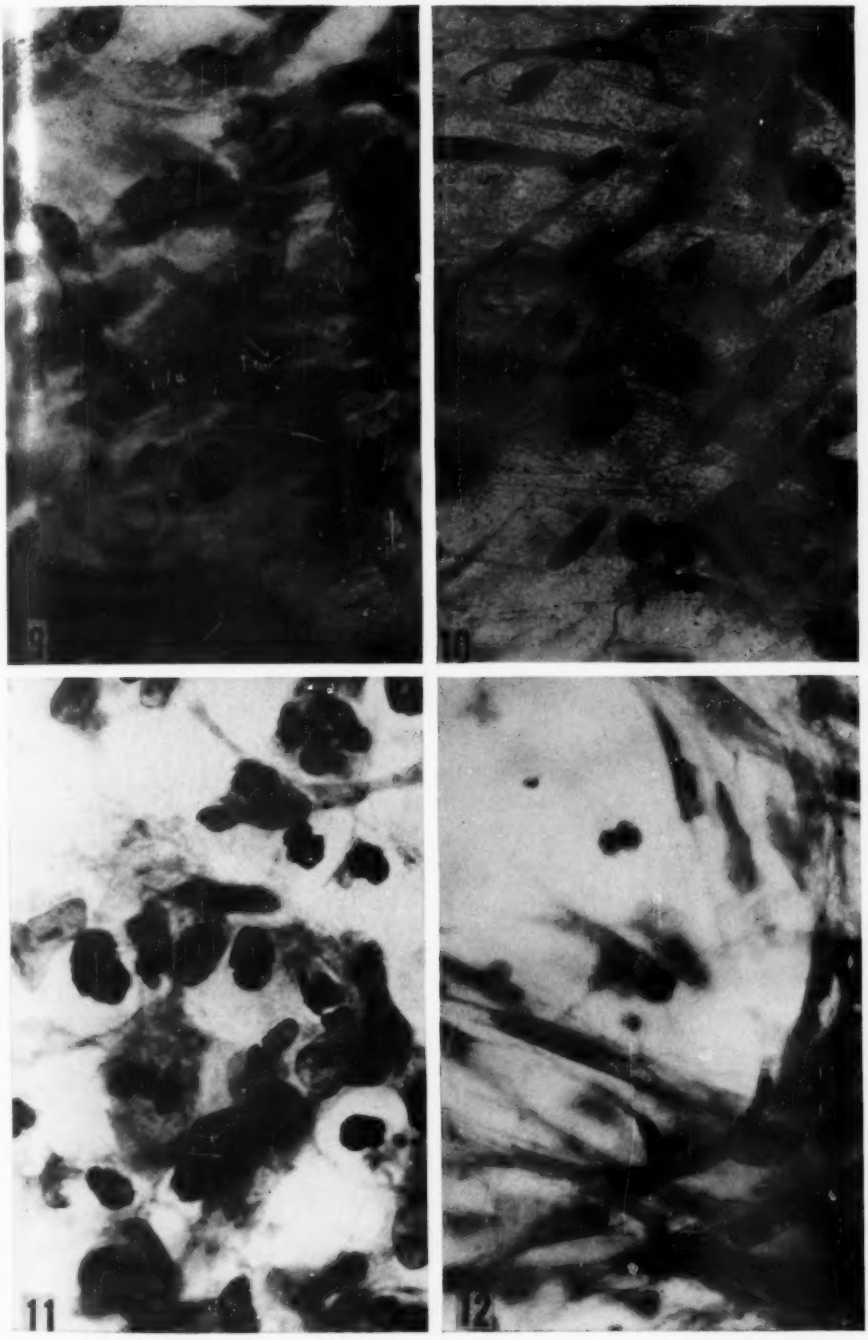
PLATE 116

FIGURE 9.—Human adrenocortical tumor after 11 days in VM chamber showing outgrowth of spindle-shaped cells and chromosomes of a dividing cell. × 800

FIGURE 10.—Cellular outgrowth from fetal human adrenal cells after 20 days in VM chamber. × 500

FIGURE 11.—Human myelogenous leukemia after 10 days in chamber. × 1,200

FIGURE 12.—Rous sarcoma cells after 10 days in VM chamber. × 500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A Method for the Cytologic Detection of Tumor Cells in Whole Blood [1]

RICHARD A. MALMGREN, JOHN C. PRUITT, PASCO R. DEL VECCHIO, *and* JOHN F. POTTER, *Pathologic Anatomy Branch, Field Investigations and Demonstrations Branch, and Surgery Branch, National Cancer Institute,[2] Bethesda, Maryland*

One of the crucial problems in the cytologic detection of cancer cells in the blood of tumor-bearing individuals has been the satisfactory preparation of the specimen to be examined.

Ideally, a relatively large volume of blood should be processed to increase the probability of finding any tumor cells. It is essential that the technique should minimize the chances of destroying the tumor cells and should not alter their morphology. Removal of a large portion of the cellular elements of the whole blood must also be accomplished without damage to the tumor cells. From a practical standpoint, the tumor cells should ultimately be concentrated in a small area to facilitate microscopic examination. It would also be desired that the preparation permit quantitating the number of tumor cells per volume of blood, and that it give some indication of the viability, clumping, and general morphologic characteristics of the cells.

In 1955, Engell (*1*) published an excellent review of this field in which he included both historical background and critical comments on the previous studies. After evaluation of the earlier work, Engell, in an attempt to overcome some of the difficulties encountered by others, employed saponin for hemolyzing the erythrocytes. He prepared sections for microscopic examination from a paraffin block of the leukocyte-tumor cell pellet held together by a fibrin clot. This method used 2 to 5 ml. of blood, and Engell estimated a 15 to 20 percent loss of tumor cells.

More recently Sandberg and Moore (*2*) have described a sedimentation technique which, when repeated in our laboratory, also resulted in a loss of some tumor cells. Clumps of tumor cells had sedimented rapidly and were lost in the red-cell pack.

Therefore, in the present study, the enzyme streptolysin-O [3] was in conjunction with a modification of Seal's (*3*) filter technique for the threefold purpose of simplifying the procedure, using a substantially larger volume of blood than had been previously used and reducing tumor cell loss and destruction to a minimum. By this procedure all the criteria

[1] Received for publication December 18, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Difco, Streptolysin-O, Difco Laboratories, Detroit, Michigan.

463225—58——19

of a satisfactory preparation were met and the objections to previously described methods were overcome.

Procedure

For the initial studies the test preparation consisted of 20 ml. of human blood plus a known number of LAC tissue-culture tumor cells [4] (*4*). The number of LAC tumor cells added was closely standardized by a number of prior countings in a hemocytometer.

The 20 ml. of whole blood plus 1,000 LAC tumor cells were then placed in a 50 ml. centrifuge tube and centrifuged at 300 × g for 25 minutes to separate the cells from the plasma. The plasma was removed by pipette and replaced with 30 ml. of 0.85 percent saline. After thorough mixing, the centrifugation was repeated at 300 × g for 25 minutes. The supernatant was removed and the cells resuspended in 30 ml. of 0.85 percent saline for a second washing.

The removal of plasma and the saline washes are essential for the removal of the naturally occurring anti-streptolysin.

Following this, the packed cells were resuspended in 20 ml. of 0.85 percent saline and 10 ml. of an isotonic phosphate buffer (*p*H 6.4) containing 500 mg.[5] of streptolysin-O was added to lyse the erythrocytes and polymorphonuclear leukocytes (*5*). The test preparation was then incubated at 39° C. for 10 minutes, with gentle shaking.

At the end of the incubation period, 217 ml. of 0.85 percent saline was added. The entire preparation was then filtered through 5μ Millipore filters [6] at 20 mm. mercury pressure as described by Seal (*3*). A new filter was used for each 50 ml. of the preparation; thus the tumor cells in the original 20 ml. of blood were evenly distributed on 5 filters.

A single glass stirring rod was used throughout the procedure. The stirring rod and all the containers were rinsed with an additional 500 ml. of 0.85 percent saline in 50 ml. aliquots and passed through a 6th filter.

The filters and filter pads were promptly placed in modified Carnoy's fixative (*6*). This fixative, by the removal of any retained hemoglobin, made microscopic examination more satisfactory. The material remained in the fixative not less than 10 minutes.

The filters, without the filter pads, were carried through the Papanicolaou stain in a Technicon nylon autopsy tray with cover. They were mounted in Permount on a 52 × 77 mm. slide. The Permount was placed on both the slide and the coverslip to seal the filter more completely. The entire slide was examined microscopically for tumor cells. Areas containing tumor cells were marked (*7*) and the number and character of cells found were recorded.

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[4] The authors wish to express their thanks to Miss Frances Y. Legallais and Dr. Alan S. Rabson who kindly provided the LAC tissue-culture cells used in this study.

[5] After the removal of anti-streptolysin in the plasma, numerous trials with different concentrations of streptolysin-O were performed and it was found that there was a moderately wide range of concentration which was effective in producing hemolysis and leukolysis. It therefore seems simpler to express the quantity of streptolysin-O used in terms of milligrams rather than the biologic nomenclature of test dose.

[6] Millipore filter #SM from Millipore Filter Corporation, Watertown, Mass.

In other studies, tumor cells were obtained from surgical and autopsy material. The tumor tissue was scraped from the cut surfaces of the neoplasm into 50 ml. 0.85 percent saline, and this suspension was added to 20 ml. whole blood for processing.

The purpose of the latter studies was to demonstrate the effects of the technique on the cells of various tumor types.

Results and Discussion

Todd (*5*) has previously described the destruction of erythrocytes and leukocytes occurring when streptolysin-O was added to normal whole blood. When streptolysin-O was added to whole blood containing tumor cells a similar hemolysis of the erythrocytes and destruction of the polymorphonuclear leukocytes resulted. However, the tumor cells remained morphologically intact.

In 3 separate preparations, 1,000 tumor cells, as determined by counts in a hemocytometer, were added to 20 ml. whole blood. After each of these preparations was carried through the entire procedure, the total number of tumor cells collected on the filters was determined. The number of tumor cells recovered per preparation was 1,035, 1,090, and 935. Allowing for a 5 to 10 percent error in the hemocytometer counting technique and pipetting procedure, a recovery rate of better than ± 10 percent is equivalent to total recovery of the tumor cells after processing.

Furthermore, no tumor cells were recovered on the filters when the original plasma and the saline washings used during the procedure were filtered.

In addition to the LAC tissue-culture tumor cells (figs. 1–4), an adenocarcinoma of the colon, a squamous-cell carcinoma of the cervix, a squamous-cell carcinoma of the lung, and an adenocarcinoma metastatic to the pleura were processed in the same manner and the tumor cells remained morphologically intact (figs. 5–12).

A blood specimen with LAC cells added was processed by the streptolysin-O technique. The product was collected on several filters. A filter stained immediately showed little evidence of mitotic activity. The remaining filters were incubated at 37° C. for 21 hours in Eagle's basal tissue-culture media (*8*) with 20 percent human serum. At the end of this time numerous cells in various stages of mitosis were observed. This would indicate that the procedure preserved not only the morphology of the cells, but also their vitality and viability (fig. 4).

The combination of streptolysin-O and the Millipore filter technique therefore seems to fulfill the requirements of an ideal procedure for preparing large volumes of blood containing tumor cells for microscopic examination. The tumor cells were concentrated with minimal cell loss and morphologic damage, and the removal of a maximal amount of the normal blood-cell elements was accomplished. The slides prepared by this procedure contained an even distribution of cells and provided an easily screenable field (fig. 1).

This procedure required 2 hours from the time the blood was collected

until the slides were ready for examination. With a multiple filtration apparatus, the time could be reduced to 1 hour and 40 minutes. Approximately 30 to 45 minutes per slide are required for the microscopic screening procedure.

This technique has also proved satisfactory for processing other body fluids for cytologic examination.

Summary

A procedure is described using streptolysin-O and a Millipore filter for preparing 20 cc. of whole blood for the cytologic detection of cancer cells. By this technique the morphology and apparent viability of the cancer cells studied are preserved and the loss of tumor cells is minimized. Erythrocytes and most of the polymorphonuclear leukocytes are destroyed, leaving tumor cells and lymphocytes on an easily screenable slide.

Addendum

Since this paper was submitted for publication, one of the authors (J. C. P.) found that centrifugation of the hemolized specimens in the presence of PVP (Plasdone C from the Antara Chemicals Division of General Analine and Film Co. of New York) with resuspension of the pellet in 0.85 percent saline may in some instances facilitate filtration and result in somewhat lessened polymorphonuclear debris.

The PVP is added to the blood after streptolysin "O" hemolysis to a final concentration of 0.7 percent. The specimen is then centrifuged at 225 $\times$ g for 15 minutes and the supernatant is pipetted off and discarded. The pellet is resuspended in 0.85 percent saline and filtered in the usual manner.

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PLATES

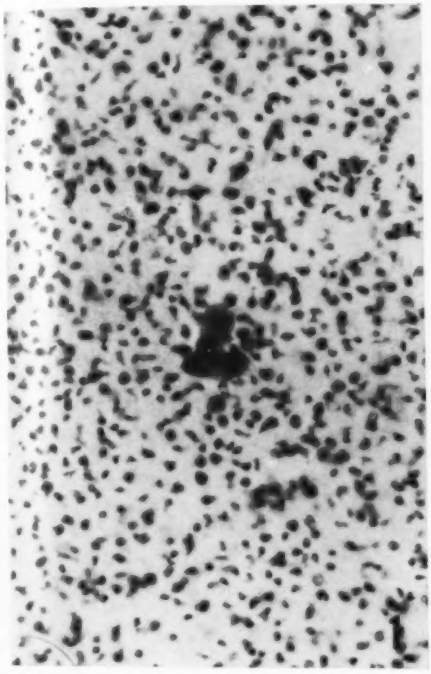
PLATE 117

FIGURE 1.—LAC tumor cells (↓) surrounded by lymphocytes and polymorphonuclear leukocyte debris. These cells are on the Millipore filters after treatment with streptolysin-O. × 325

FIGURE 2.—LAC tumor cells on Millipore filter after treatment with streptolysin-O. × 1,220

FIGURE 3.—LAC tumor cell on Millipore filter before treatment with streptolysin-O. × 1,220

FIGURE 4.—LAC tumor cells on Millipore filter after streptolysin-O treatment and incubation at 37° C. for 21 hours in tissue-culture media. Note the cells in mitosis. × 610



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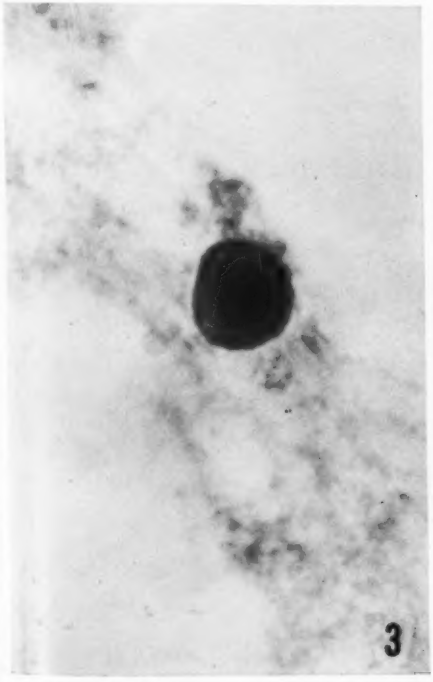
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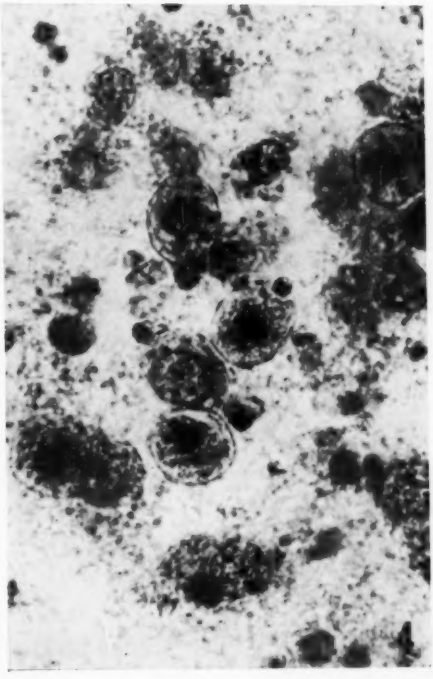
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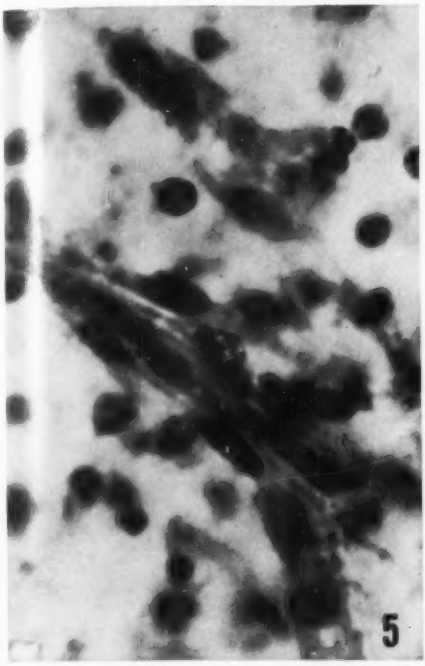
PLATE 118

FIGURE 5.—Cells from squamous-cell carcinoma of cervix treated with streptolysin-O. × 1,220

FIGURE 6.—Cells from squamous-cell carcinoma of cervix (same case as fig. 5) before streptolysin-O treatment. × 780

FIGURE 7.—Cells from squamous-cell carcinoma of the lung on Millipore filter after streptolysin-O technique. × 665

FIGURE 8.—Cell from squamous-cell carcinoma of lung (same case as fig. 7) on Millipore filter before streptolysin-O treatment. × 830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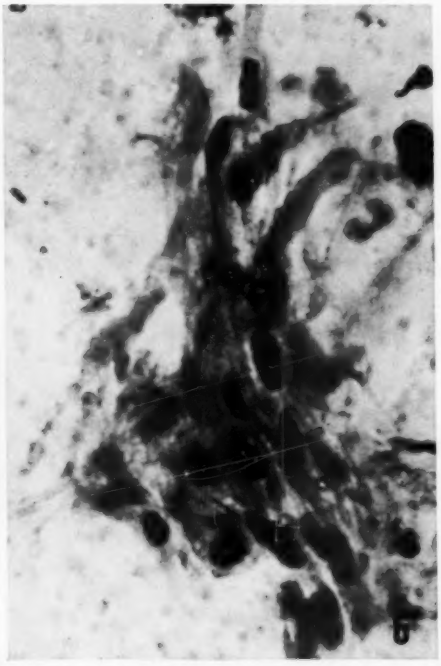
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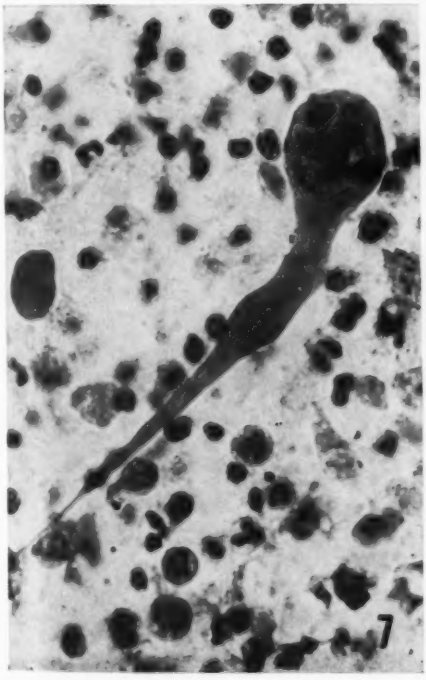
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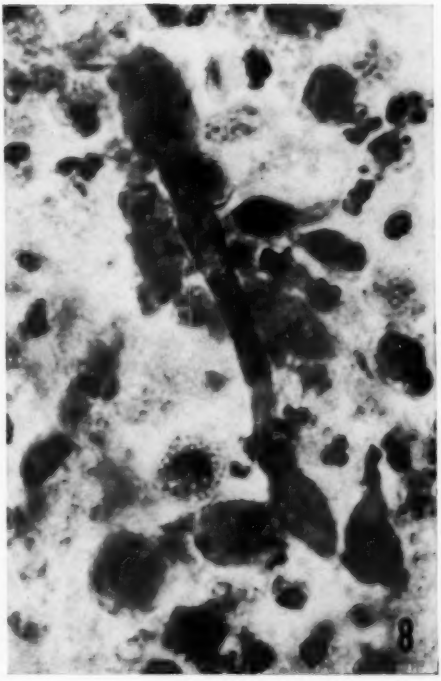
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PLATE 119

FIGURE 9.—Adenocarcinoma cells from pleural metastasis on Millipore filter after streptolysin-O technique. × 1,220

FIGURE 10.—Adenocarcinoma cells from pleural metastasis (same case as fig. 9) on Millipore filter before streptolysin-O. × 1,220

FIGURE 11.—Cells from adenocarcinoma of the colon on Millipore filter after streptolysin-O technique. × 1,220

FIGURE 12.—Cells from adenocarcinoma of colon on Millipore filter before streptolysin-O technique (same case as fig. 11). × 1,220

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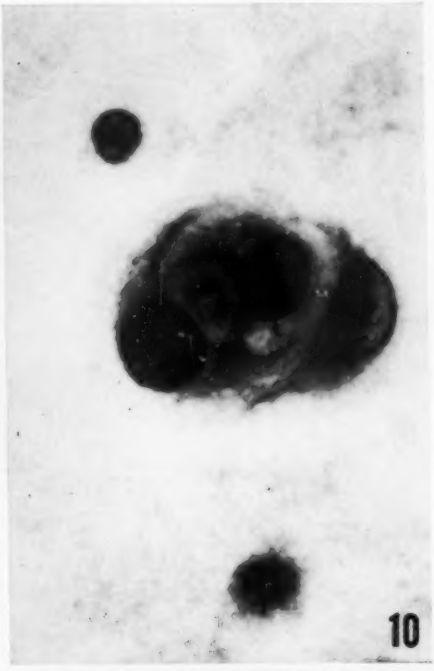
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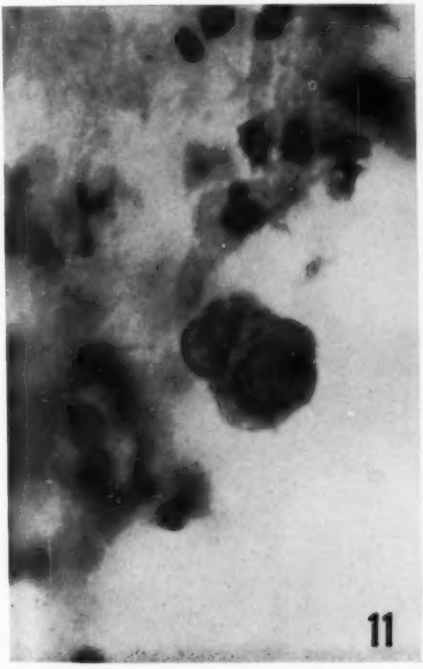
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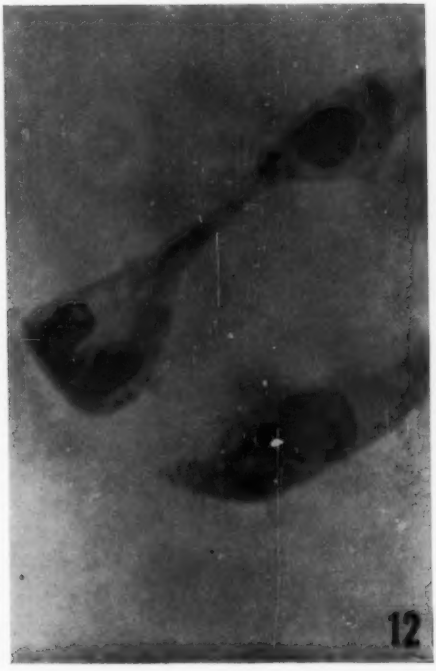
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Absence of Neutralization of Rous Sarcoma Virus by Antinormal-Chicken-Embryo Serum and Complement [1,2]

TIBOR BORSOS,[3,4] *Department of Pathobiology, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland*

Neutralization of Rous sarcoma virus (RSV) with antinormal-chicken tissue (adult or embryonic) sera, as well as with specific antiviral sera from infected birds, has been reported many times (*1*, *2*). Recent advances in virus assay, however, permit the reinvestigation of the neutralization of the virus by different antisera. In the experiments described in this paper, two assay techniques have been used: 1) the counting of tumor lesions produced on the chorio-allantoic membranes of chick embryos (*3*, *4*), hereafter referred to as the CAM method; 2) the counting of hemorrhagic lesions in the chick embryos (*5–7*), referred to as the intravenous (IV) method.

The first method utilizes the tumor-producing capacity of the virus. The ectodermal lesions are small tumors, and the growth of susceptible cells is used to detect virus activity. Presumably interference with cell multiplication will reduce the number or the size of the tumors.

The second method utilizes the, presumably, destructive activity of the virus (*8*). Susceptible endothelial cells are thought to be destroyed by virus activity, and the resulting lesion is a hemorrhage.

In both methods the numbers of lesions produced are directly related to the dilution of the virus (*4*, *7*).

Rubin (*1*), using the CAM method, concluded that antinormal-tissue serum and complement interfered with the multiplication of infected cells and thus apparently neutralized virus activity; therefore, the virus did not have ". . . unaltered normal cell protein (as) an integral part of the functional surface of the Rous sarcoma virus." Observations on a host virus system, which is expressed by destructive lesions, subjected these conclusions to further testing.

Materials and Methods

Virus.—The strain of RSV was originally obtained from Dr. W. R. Bryan, of the National Cancer Institute, and was in its eleventh passage

[1] Received for publication December 18, 1957.

[2] This work was supported by grant C-1230 from the National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Predoctoral Research Fellow, National Cancer Institute, Public Health Service.

[4] I wish to acknowledge the valuable help and criticism of Dr. F. B. Bang during this investigation.

463225—58——20

through the liver of chick embryos (*6*). The virus was kept in citrate buffer (*9*) in an electric deepfreeze at −40° C.

Sera.—Rabbit-antinormal-chick-embryo-tissue serum (Ra-aNCS) was prepared as described in (*1*). Forssman antibody, prepared in rabbits against boiled sheep red-blood-cell stromata, was obtained from Dr. H. J. Rapp, Department of Microbiology, of this school, who also supplied the fresh-frozen guinea-pig serum as a source of complement (C′). Neutralization tests carried out on this batch of C′ showed no trace of anti-RSV activity in either system; therefore, no C′ controls as such were included in the experiments. Chicken anti-Rous-virus serum was obtained from a bird (#46) that had carried a large tumor in its left pectoral muscle for 6 weeks. Heat inactivation of sera was carried out in a water bath at 56° C. for 30 minutes.

Embryos.—White Leghorn embryos were used in all experiments. They were incubated for 11 days in a Jamesway single-stage incubator at 37.5 C., with a relative humidity of 86 percent. The eggs were turned automatically three times daily. For intravenous (IV) inoculation, the embryos were prepared as described by Lo and Bang (*6*); harvesting occurred 7 days after inoculation and the total number of hemorrhagic lesions per egg was recorded. For CAM inoculation, the embryos were prepared by a technique slightly modified from that of Rubin. Embryos were marked over the membrane for drilling and a small slit about 3 mm. in length was made. After the air sac was punctured with a needle stuck through a rubber stopper, gentle suction was applied to the hole in the air sac, and the shell membrane was pricked at the slit with a sterile needle. The operation was performed while the egg was being candled, thus permitting continuous observation of the separation of the CAM from the shell membrane. Inoculation was carried out by inserting a 27-gauge, ½-inch needle halfway through the slit and delivering 0.05 ml. from the attached tuberculin syringe. Harvesting membranes and counting lesions were done as described by Rubin (*4*).

Neutralization test.—Appropriate dilutions of sera and virus were mixed in phosphate buffer and each mixture was incubated for about 1 hour at 4° C. before inoculation. Each embryo received 0.05 ml. of the inoculum regardless of route of inoculation; 15 embryos per inoculum were used.

Results

Comparative titration of Rous sarcoma virus: intravenous and chorioallantoic membrane systems.—The only comparative assay of this virus was reported by Rubin (*4*). He assayed the same stock of virus simultaneously on the CAM of embryos, and subcutaneously in young chicks. He found that the CAM method was more reliable and more efficient. Two experiments were designed to explore the comparative efficiency of the two methods; in experiment A, serial tenfold dilutions of the virus were inoculated; in experiment B, only one dilution was prepared and inoculated. The results of these experiments are recorded in table 1. The data indicate that the two systems are directly comparable. There was

TABLE 1.—*Comparative assay of Rous sarcoma virus on the chorio-allantoic membrane and in the vascular system of chick embryos*

Experiment	Route	Dilution 10^{-3}	Dilution 10^{-4}	Dilution 10^{-5}	
A	Intravenous	67	7. 2	0. 7	Average no. of lesions per embryo; 15 embryos per inoculum.
	CAM	75	8. 2	0. 8	
		Dilution $10^{-3.5}$		S.E.*	
B	Intravenous	23. 9		±1. 9	Average no. of lesions per embryo; 30 embryos per inoculum.
	CAM	27. 0		±2. 3	

*S.E. = standard error.

close agreement in the number of hemorrhagic lesions per embryo and of the tumorous lesions. However, every time a direct comparison was attempted, the number of tumors slightly exceeded the number of hemorrhagic lesions. This might be due to several factors: *a*) some of the hemorrhagic lesions could have been destroyed before counting, *b*) the membranes might have shown nonspecific lesions (*10*), or *c*) epithelial cells might be more susceptible than endothelial cells.

Effect of heated rabbit-antinormal-chicken-embryo serum and complement.—It is evident from the previous experiments that the two systems can be directly compared. The following experiment was designed to investigate the effects of antitissue serum on the virus in both systems. Three tubes were set up: *Tube A* received 0.5 ml. of heated Ra-aNCS, diluted 1:5, 0.2 ml. of heated human cord serum (HCS), which has no anti-RSV activity, and 0.3 ml. of virus containing 600 hemorrhagic units (HU). *Tube B* received 0.5 ml. of heated antiserum, 1:5, 0.2 ml. of C′, and 0.3 ml. of virus (600 HU). *Tube C* served as control with 0.7 ml. of HCS, diluted 1:7, and 0.3 ml. of virus (600 HU). Results presented in table 2 show that only the antiserum-C′ combination is able to reduce virus activity, and only in the CAM system.

TABLE 2.—*Effect of heated rabbit-antinormal-chicken-embryo serum and complement on Rous sarcoma virus**

Experiment	Tube:	*A* Antiserum 1:10; virus 600 HU	*B* Antiserum 1:10; virus 600 HU; complement 1:5	*C* Human cord serum 1:10; virus 600 HU
	Number of lesions:			
I	Intravenous	22. 8	26. 0	25. 4
	CAM	29. 0	1. 7	30. 4
II	Intravenous	31. 5	26. 6	35. 0
	CAM	34. 0	1. 2	40. 0

*HU = hemorrhagic units.

TABLE 3.—*Effect of unheated rabbit-antinormal-chicken-embryo serum on Rous sarcoma virus**

Experiment	Tube:	*A*	*B*
		Antiserum 1:10; virus 600 HU	Human cord serum 1:10; virus 600 HU
	Number of lesions:		
I	Intravenous	24. 2	26. 0
	CAM	5. 0	33. 4
II	Intravenous	24. 2	30. 6
	CAM	4. 6	33. 4

*HU = hemorrhagic units.

Effect of unheated rabbit-antinormal-chicken-embryo serum.—To test the effect of C′ in unheated rabbit serum the following experiment was set up: *Tube A* received 0.5 ml. of unheated antiserum, diluted 1:5, and 0.5 ml. of virus containing 600 HU. *Tube B* was the HCS control with 0.5 ml. of serum, 1:5 and 0.5 ml. of virus (600 HU). Table 3 presents the results. It is seen that unheated antiserum reduces the number of tumors on the membrane but has no effect on the number of hemorrhagic lesions produced. It is probable that the native C′ is acting in this case.

Effect of Forssman antibody and complement.—The previously described experiments indicate that the number of hemorrhagic lesions was not reduced by antitissue serum and C′ but that the number of tumors was greatly reduced by this serum combination. Rubin has shown that Forssman antibody also reduced the number of tumors on the membrane. It was considered possible that, since chick red blood cells contain Forssman antigen, the presence of large amounts of antibody might injure the host thus altering its responsiveness. The following experiment was set up to test this point: *Tube A* received 0.5 ml. of Forssman antiserum, diluted 1:5, 0.2 ml. of HCS, and 0.3 ml. of virus (600 HU). *Tube B*

TABLE 4.—*Effect of Forssman antibody and complement on Rous sarcoma virus**

Experiment	Tube:	*A*	*B*	*C*
		Forssman antibody 1:10; virus 600 HU	Forssman antibody 1:10; virus 600 HU; complement 1:5	Human cord serum 1:10; virus 600 HU
	Number of lesions:			
I	Intravenous	25. 6	23. 2	24. 0
	CAM	31. 0	3. 4	30. 9
II	Intravenous	27. 5	34. 6	25. 6
	CAM	33. 4	2. 4	31. 5

*HU = hemorrhagic units.

received 0.5 ml. of the antiserum, 1:5, 0.2 ml. of C′, and the same virus as A; C served as the usual HCS control. Table 4 summarizes the results of this experiment. It is seen that only the CAM system is affected; if there is any damage to the circulating system of the embryo by the Forssman antibody-C′ mixture, it does not affect the titer of the virus.

Effect of specific antibody and complement.—The last set of experiments explored the effect of C′ acting together with antiviral chick serum. Rubin's experiments showed that such a combination did not enhance the neutralizing powers of the chick serum in the CAM system. As expected, it had no effect when the virus-serum mixture was inoculated intravenously. The experiment was set up as follows: *Tube A* contained 0.5 ml. of antiviral serum, diluted 1:5, 0.2 ml. of HCS, and 0.3 ml. of virus containing 600 HU. *Tube B* received the same amount of antibody, 0.2 ml. of C′, and the same amount of virus. *Tube C* contained the usual HCS control. Table 5 records the results. C′ does not affect neutralization in either system; moreover, neutralization occurs in both systems to about the same extent.

TABLE 5.—*Effect of specific antiviral antibody complement on Rous sarcoma virus**

Experiment	Tube:	*A* Antiserum 1:100; virus 600 HU	*B* Antiserum 1:100; virus 600 HU; complement 1:5	*C* Human cord serum 1:10; virus 600 HU
	Number of lesions:			
I	Intravenous	1.5	2.0	27.0
	CAM	3.0	3.4	38.0
II	Intravenous	1.2	not done	23.5
	CAM	1.7	not done	31.5

*HU = hemorrhagic units.

Discussion and Conclusions

The experiments reported in this paper give further support to Rubin's findings that the apparent neutralization of RSV by antinormal-chick-embryo-cell antibodies is due to an impairment of the ability of cells to multiply. It is evident that the actual virus titer is not reduced by anti-embryonic-cell serum in the hemorrhagic system. To a limited extent, this might be due to a necessary dilution of the antibody before inoculation into the circulating system of the embryo. Attempts were made, in experiments not reported here, to inoculate very large amounts of Forssman antibody by repeated injection into the circulation of the embryo. None of the experiments indicated a reduction in the number of hemorrhagic lesions, which suggests that dilution following intravenous injection has little or no effect. Red blood cells might also contribute to removing the antibody before reaching susceptible cells; further experiments would be

necessary on this point. In any event, the experiments strongly suggest a lack of association between the infectious unit of the virus and the anti-embryo-cell antibody.

Moreover, these experiments give further support to Duran-Reynals' experiments (*5*) in which he showed the identity of the tumor-producing and hemorrhagic-lesion-producing principles. It would be rather unlikely that two different viruses would give almost identical infectious unit counts in two rather dissimilar systems and also would be neutralized to the same extent by antibody.

The destructive property of a tumor virus might prove very useful in future experiments. Groupé *et al.* (*11*) recently reported that RSV, which has been passed in the brain of young chicks, seemed altered in some of its properties. Specifically, the virus started to produce hemorrhage and liver lesions; it had not done so in previous passages. They also reported that the virus tended to be in high concentrations in the liver in the later passages. It would be of great interest to explore the different passage-virus lots, before and after the alteration occurred, with the two methods of titration described in this paper.

Summary

Comparative assays of Rous sarcoma virus, as measured by the production of tumors on the chorio-allantoic membrane of chick embryos and the production of hemorrhagic lesions in chick embryos, were carried out. It was found that the number of tumors and hemorrhagic lesions produced in the two systems are approximately equal for the same virus suspensions.

No neutralization of Rous sarcoma virus was detected in the hemorrhagic system except with antiviral serum obtained from convalescent birds. In contrast, the number of tumors on the chorio-allantoic membrane was reduced both by the antiviral serum and by antinormal-chicken-embryo-tissue serum and complement.

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Neoplasms in Mice Inoculated with a Tumor Agent Carried in Tissue Culture [1,2]

SARAH E. STEWART,[3] BERNICE E. EDDY,[4] *and* NINETTE BORGESE,[3,5] *National Cancer Institute, and Division of Biologics Standards,*[6] *Bethesda, Maryland*

The induction of neoplasms in mice inoculated shortly after birth with cell-free extracts or filtrates prepared from mouse tumors has been reported by several investigators (*1–9*). In this laboratory, pleomorphic tumors of the parotid glands were commonly produced with such materials. Cell-free extracts prepared from the parotid-gland tumors did not induce neoplasms in mice that were inoculated within 24 hours after birth. We were able, however, to demonstrate the release of a tumor-inducing substance from such neoplasms by the use of tissue culture (*10*). Monkey-kidney tissue cultures were inoculated with mouse leukemia or parotid-gland tumor preparations and incubated at 36° C. for 1 to 2 weeks. A tumor-inducing agent which could be passed serially *in vitro* was demonstrated in the supernatant fluids from 8 of 28 such tumor preparations.

In the work reported here, tissue cultures prepared from minced, mouse embryo cells were found to be more effective than monkey kidney cells for the propagation of the tumor-producing agent as shown by: 1) a greater frequency of certain primary tumors in the injected mice; 2) a shortening of the latent period or the time required for appearance of the tumors after inoculation; 3) successive passage of five sublines of the agent in tissue culture, one of which is now in its 29th passage; 4) tumor-inducing activity of the agent at dilutions of 10^{-5} and occasionally at 10^{-7}.

Materials and Methods

Tissue Culture

Mouse-embryo tissue cultures were prepared as described previously (*11*) and were inoculated by one of two methods: 1) adding a 5 mm. loopful of finely minced tumor tissue; or 2) adding 1 ml. of the supernatant fluid, which was obtained by centrifugation (10 minutes at 3000 r.p.m. at

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[3] National Cancer Institute.

[4] Division of Biologics Standards.

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0° C. in an International refrigerated centrifuge) of a 10 to 20 percent suspension prepared by grinding the weighed tumor tissue with sterile alundum and diluting with Earle's balance salt solution (*12*). Tumor cells were introduced into the tissue culture by these methods. In one experiment a "cell-free" extract was prepared from #60 leukemia, which was used as the culture inoculum (*see* sublines of tumor agent). Uninoculated control cultures of mouse embryo were maintained with each experiment. All cultures were incubated at 36° C.

The cultures were observed for cytopathic changes and the nutrient fluid was removed and replaced at weekly intervals. Only fluids from cultures that had been maintained 2 weeks or longer were transferred to new cultures or were used to inoculate animals. If the cultures were less than 2 weeks old the amount of tumor agent was frequently not enough to elicit tumors (*10*). Tissue-culture passages were made by pipetting 2 ml. of fluid from a culture directly to a new culture bottle containing a sheet of cells in 8 ml. of the maintenance medium.

Control Tissue Cultures

Uninoculated tissue cultures from each lot of mouse embryo tissues were kept at 36° C. and the supernatant fluids transferred in the same manner as the inoculated cultures. Supernatant fluids from each passage were also injected subcutaneously into newborn mice of the same types that had received the inoculated cultures.

Inoculum

The tissue-culture supernatant fluids used for inoculating newborn mice were refrigerated at 4° C. immediately after harvesting. In most instances they were administered to test animals within 24 hours of harvesting, after the cells and cell debris had settled. When large amounts of cells were present, the culture fluids were centrifuged for 15 minutes at 3000 r.p.m. at 0° C. and the supernatant fluid was used for the inoculum. Subline 3919^{-3} was filtered through a bacteria-proof Selas .03 filter prior to inoculation.

The inoculum in all instances was 0.1 ml. It was injected subcutaneously with a 1-inch, 27-gauge hypodermic needle, which was introduced in the back at the base of the tail, and the fluid deposited in the tissues about the head and axillae. As the needle was withdrawn, the skin about the puncture was compressed long enough to prevent the inoculum from leaking. Each injected group of newborn mice was kept in a sterile dish for 15 to 20 minutes before being returned to the mother since this procedure tended to prevent leakage of the inoculum.

Mice

Strain C3Hf/HeN mice and randomly bred Swiss mice were procured from the animal production facilities of the National Institutes of Health. Strain AKR mice were obtained from the Roscoe B. Jackson Memorial

Laboratory, Bar Harbor, Maine. Hybrid (C3Hf × AKR)F_1 mice were obtained by mating C3Hf females with AKR males. All mice inoculated were less than 1 day old. Individual mice of each litter were randomized so the groups receiving different inocula were as near alike as possible. The mice were housed in plastic cages, 6 to 10 in a cage, depending on the size of the litters, fed Purina laboratory chow, and given tap water *ad libitum*. The sexes were not separated and their progenies were discarded shortly after birth, except in those instances where they were kept for observation to determine whether the progeny developed tumors. They were observed for development of gross tumors and allowed to live as long as possible after the tumors appeared.

Tissues from all animals that died or were killed were fixed in Tellyesniczky's formalin-acid alcohol fluid (*13*), embedded, sectioned, and stained with hematoxylin-eosin for histological examination.[7]

Sublines of Tumor Agent

Extracts or homogenates from 5 tumors were cultured, and the tumor agent was carried through 5 to 29 serial passages. The origins of 4 of the tumors, #3469, #3919, #60, and #3791, have been described (*10, 11, 14*). A brief review of these tumors and the origin of #3607 follows:

Tumor #3469.—Monkey kidney cultures were inoculated with minced tumor tissue from an AKR mouse, #24424, with spontaneous leukemia. Newborn (C3Hf × AKR)F_1 hybrid mice were inoculated with a second-passage fluid from these cultures. One of the mice, #3469, that developed tumors was killed and a mince from the parotid-gland tumors was added to monkey kidney cultures (*14*). Two passages were made in monkey-kidney tissue cultures and subsequent passages were carried in mouse-embryo tissue cultures. This tumor-agent subline is at present in its 29th serial passage.

Tumor #3919.—Fourth-passage tissue-culture fluid from #3469 was injected subcutaneously into newborn (C3Hf × AKR)F_1 hybrid mice. Twenty-nine days later, two of these, free from gross tumors, were killed. Their livers, spleens, and parotid glands were minced and added to mouse-embryo tissue cultures. Third-passage fluid from these cultures was injected into newborn mice. One mouse, #3919, that developed tumors was killed, and a cell suspension prepared from the parotid-gland tumors was added to mouse embryo cultures. Newborn mice were inoculated with fluids from the second, third, and fifth tissue-culture passage.

Leukemia #60.—This tumor, which is a transplanted AK/n leukemia that grows well in AKR mice, has repeatedly been a good source of tumor agent (*15*). As described earlier (*14*), a cell-free extract prepared from the 92nd transplant passage in AKR mice was inoculated into monkey-kidney cell tissue cultures. A second passage was made in monkey-kidney tissue cultures and subsequent passages were carried in mouse embryo cultures. The fourth-passage tissue culture became contaminated

[7] Acknowledgment is made of the valuable assistance of Joseph M. Albrecht and associates in preparing the tissues for histological examination.

with bacteria and was filtered through a Seitz S_3 filter for use in the next passage. Supernatant fluids through the eighth serial passages were inoculated into newborn (C3Hf × AKR)F_1 hybrid mice. Tenth-passage material was used for serial dilutions in Swiss mice.

Tumor #3791.—Hybrid (C3Hf × AKR)F_1 newborn mice were inoculated with fluid from a mouse embryo culture which had received a cell-free extract from mouse leukemia #60 (*14*). One mouse, #3791, that developed tumors was killed. A thymoma was removed, minced, and put into mouse embryo culture and the supernatant fluids were carried in serial passage. Cytopathic changes, as described earlier (*11*), were noted in the culture of the first passage during the 4th week of incubation and during the 3rd and 4th weeks of 3 subsequent passages. Supernatant fluids from these cultures killed many of the inoculated mice within 14 days. By the fifth passage the cytopathic changes had disappeared and few mice receiving this material died early after inoculation. The tumor agent is now in its 18th-passage generation.

Tumor #3607.—A hybrid (C3Hf × AKR)F_1 mouse that had been inoculated when newborn with a cell-free extract from a spontaneous AKR leukemia developed a paraganglioma in the parotid-gland area. A cell-free extract, prepared from the paraganglioma, induced neoplasms when inoculated into newborn mice. One of these mice, #2816, which developed parotid-gland tumors, was killed when 10 months old, and a mince prepared from the tumors was inoculated into monkey kidney culture (*10*). Newborn mice inoculated with fluid from the second passage in tissue culture developed tumors. One of them, #3191, was sacrificed when 3 months old and a mince prepared from the parotid-gland tumors was put into monkey tissue cultures. Second-passage fluid from these cultures produced tumors when inoculated into newborn mice. A mouse, #3607, with parotid-gland tumors was killed at 4 months and portions of the tumors were ground in alundum and diluted in Earle's balanced salt solution to make a 20 percent suspension. Supernatant fluid from this tumor suspension was added to mouse-embryo tissue cultures. Cytopathic changes like those described for #3791 (*11*) were noted in these cultures and in 3 subsequent passages. Very few mice survived inoculation with fluids from these first 4 passages. As with #3791 the cytopathic changes disappeared by the fifth passage and few mice receiving this material died early after inoculation.

Cell-Free Extracts from Leukemia #60 Inoculated into Newborn Swiss Mice

Although cell-free extracts of leukemia #60 were shown to induce neoplasms when inoculated into newborn strain C3H and (C3Hf × AKR)F_1 hybrid mice, their activity in newborn Swiss mice was not known. A cell-free extract was prepared from the 114th passage of leukemia #60 by the method already described (*3*), and newborn Swiss mice and (C3Hf × AKR)F_1 hybrids were inoculated subcutaneously with 0.1 ml. of the extract.

Titration of Tumor Agent

Supernatant fluid from 2-week-old cultures of first passage of subline #60 in monkey kidney cells was diluted tenfold through 10^{-5} in Earle's balanced salt solution. One ml. each of undiluted, 10^{-1}, 10^{-3}, and 10^{-5} was inoculated into each of two bottles of monkey-kidney cell cultures and incubated for 2 weeks at 36° C. Supernatant fluids from these cultures were inoculated into newborn Swiss mice to determine the dilutions of tumor agent which would induce neoplasms after reculture of the dilution in the monkey kidney cells. Tenth-passage material of #60 subline and thirteenth passage of #3469, both from mouse-embryo cell cultures, were tested in the same manner by reculturing the undiluted and tenfold dilutions from 10^{-1} through 10^{-8} in mouse-embryo tissue cultures before inoculating them into newborn mice.

Eighth-passage material from #60 and fifth-passage material from #3607 sublines, both from mouse-embryo cell cultures, were titrated for tumor induction without reculturing the dilutions before inoculating them into the mice.

Results

Six litters of (C3Hf × AKR)F_1 hybrids (36 mice) and 6 litters of Swiss mice (50 mice), which were inoculated when newborn with cell-free extract of leukemia #60, have remained free of neoplasms during an 8-month observation period, except for 4 of the hybrid mice which developed early lymphocytic leukemia and 1 Swiss mouse which developed parotid-gland tumors and an osteogenic sarcoma of the scapula when 7.5 months old. Although this particular preparation from leukemia #60 appears to have been low in tumor agent as seen by the low incidence of induced tumors in the hybrid mice, there was sufficient agent present to show that Swiss mice are susceptible to the agent without first passing it in tissue culture.

The results on the serial passages of the 5 sublines are given in table 1. All mice listed were autopsied and examined for gross tumors, but not all have as yet been examined histologically. Of 211 mice inoculated with undiluted fluids from tissue-culture passages of the 5 different sublines of tumor agent, 179 survived 2 months or longer. One hundred and twelve of these were (C3Hf × AKR)F_1 hybrid mice. Seventy-seven, or 67 percent, developed tumors and the mean age for appearance of the neoplasms ranged from 4 to 8 months for the different passages. Sixty-seven of the surviving mice were Swiss; 62, or 92.5 percent, developed tumors and the mean age for appearance of the neoplasms ranged from 3 to 4 months. Filtration through a Selas .03 filter, known to hold back *Escherichia coli*, did not change appreciably the potency of the tumor agent, as shown by third-passage fluid of subline #3919. Titrations were not made with the filtered material but undiluted filtrate produced neoplasms in 100 percent of the mice inoculated. Cultures #3469 and #60, which were filtered through S_3 Seitz filters at their sixth and fourth passages, respectively, retained tumor-inducing activity as shown on further passages.

TABLE 1.—*Neoplasms in mice inoculated with tumor agent carried in tissue culture**

Subline tumor agent	Passage in tissue culture	Mouse strain	Mice inoculated	Mice surviving inoculum (*over 2 (months)*	Mice with tumors	Percent	Range in latent period (*months*)	Mean age at which tumors appeared (*months*)
#3469†	2	(C3Hf × AKR)F_1	19	17	11	64	2–11	7
	4	"	9	6	4	66	1–6. 5	5
	5	"	8	4	4	100	3–7	5
	6	"	20	18	13	72	1–7. 5	5
	8	"	6	6	6	100	3–7	5
	9	"	8	6	4	66	5. 5–9	5
	13	Swiss	18	14	12	86	2. 5–6	4
#60‡	1	(C3Hf × AKR)F_1	12	8	6	75	3–9	6
	2	"	8	5	5	100	3–12	6
	3	"	8	7	7	100	2. 5–7	5
	5	"	12	10	5	50	7. 5–10	8
	6	"	18	15	7	49	4. 5–8	6
	7	"	10	7	3	43	4–7	4
	8	"	4	3	2	66	4–10	4
#3607	5	Swiss	15	10	10	100	2–4. 5	3
#3791	5	Swiss	25	19	17	89	2–6	3
#3919	2	Swiss	9	7	7	100	2–6	3
	5	"	15	11	10	90	2–6	3
#3919 (filtered) §	3	Swiss	7	6	6	100	2–6	5

*All passages were in mouse embryo, except 1 and 2 of #60 and #3469 which were in monkey-kidney tissue cultures.
†Cultures filtered through Seitz filter at sixth passage.
‡Cultures filtered through Seitz filter at fourth passage.
§Culture supernatant fluid filtered before inoculating mice (.03 Selas filter).

The anatomical types of neoplasms and their frequency of occurrence produced by the five sublines of tumor agent are listed in table 2. Some of the mice listed received material from the dilution experiment. No detailed study has as yet been made to determine whether dilution of the agent alters the response as to types of neoplasms induced. In those mice that have been examined histologically there appears to be no difference. Every mouse had at least 1 pleomorphic tumor of the parotid glands, and many had additional primary neoplasms, sometimes as many as 8. Tumors of the salivary glands were usually the first to be detected during life, but occasionally osteogenic sarcomas or mammary neoplasms appeared earlier. No reasonable idea of the onset of the internally located cancers was obtained. There were no differences in the types of tumors observed in the Swiss and the (C3Hf × AKR)F_1 hybrid mice examined, except that the papillary pleural lesion was seen only in the Swiss mice.

Pleomorphic tumors of salivary glands, epithelial thymomas, spindle-cell sarcomas, and carcinomas of the adrenal medulla have been found in mice injected during the 1st day of life with simple extracts made from several different experimental tumors. The neoplasms of the same sites found in these experiments conform in their structural characteristics to the previous description (*10*) as the angiomatous, pulmonary, and

TABLE 2.—*Frequency and types of tumors and lesions observed on histological examination of the inoculated mice*

Subline of tumor agent inoculated	#3469	#60	#3607	#3791	#3919
Number of mice examined	36	40	25	8	8
Pleomorphic tumors of mucous glands:					
Parotid glands	36	40	25	8	8
Submaxillary glands	15	13	19	3	5
Sublingual glands	4	6	2	1	1
Tracheal glands	2	4	—	2	3
Harderian glands	1	1	0	0	1
Epithelial thymomas	20	16	6	5	1
Mammary adenocarcinomas in female	9	10	2	3	2
Mammary adenocarcinomas in male	4	2	2	1	2
Sweat-gland adenocarcinomas	0	0	0	0	1
Sarcomas:					
Renal	12	3	2	2	3
Bone	5	3	0	2	1
Other locations	4	3	0	1	0
Epidermoid carcinomas:					
Hair follicles, skin	5	2	4	2	2
Mandible	1	0	0	0	0
Stomach	1	1	0	0	0
Hemangioendotheliomas, subcutaneous	7	2	2	1	0
Liver hemangioma	1	1	0	0	0
Adrenal medullary tumors	5	4	2	2	2
Primary lung tumors	1	1	0	0	0
Papillary pleural lesion	3	4	3	3	4
Papillary lesion of epicardium	1	0	2	1	1
Thyroid-gland lesion	4	5	—	3	2
Kidney convoluted tubule lesion	35	27	15	3	6

harderian-gland tumors do, which arise spontaneously or can be induced in mice by chemically defined carcinogens. The sarcomas observed in our inoculated mice were most common in the kidneys and the skeleton. In the kidneys they generally occurred in the pelvis and produced an obstruction which resulted in hydronephrosis (fig. 1). The osteogenic sarcomas were most common in the spine but were observed throughout the skeleton and were often multiple.

Mammary carcinomas have also been seen in mice receiving extracts of experimental leukemias and, in general, have conformed to types that arise spontaneously in various strains of mice, as described by Dunn (*16*). Some of the mammary tumors found in mice receiving the tissue-culture supernatant fluids have a different architecture from the conventional murine carcinomas of the breast (fig. 2) and, more importantly, were found in both sexes (fig. 3), whereas mammary cancer in mice has heretofore been limited to females in the absence of known estrogenic stimulation. This particular phase of the subject requires detailed investigation beyond the scope of this paper.

Tumors of the submucous glands of the trachea may have been more numerous than recorded. They were first discovered in routine histological sections where they reproduced in miniature the salient features of the pleomorphic salivary-gland neoplasms (fig. 4). Inspection of the trachea reveals white globular masses as much as 1 mm. in diameter distorting the normally smooth lining.

The epidermoid carcinomas of the forestomach and oral mucosa, the latter invading the mandible, presented no unusual features, but those occurring in the skin appear to be unique in our experience. These papular lesions were most prominent over the ventral body surface but sometimes extended onto the flanks or legs and seldom involved the back (fig. 5). The papules were pale yellow, almost never ulcerated, and occurred in groups that tended to be confluent. Histological sections revealed bizarre changes in the hair follicles ranging from severe degeneration and atrophy through hyperplasia to the cytological characteristics of epidermoid carcinoma (fig. 6). All of these changes could sometimes be seen in one small area of an individual section. At least one of these carcinomas has produced pulmonary metastases (fig. 7) and some have clearly infiltrated adjacent tissues. This unique lesion also requires additional study.

Adenocarcinomas of the sweat glands were found in two paws of one mouse (fig. 8).

Table 2 also lists several lesions of uncertain nature. Most abundant among them is a hyperplastic lesion affecting epithelium of convoluted tubules in the kidney. The major features have already been described (*10*) but, in the series of mice reported here, two instances of extreme dilatation of the tubule with pronounced papillary infolding of the epithelium into the lumens were found (fig. 9). Papillary lesions of the pleura and pericardium were characterized by fingerlike projections of flattened cells continuous with those of the pleura or pericardium supported by a

delicate framework of connective tissue (fig. 10). These lesions were usually discrete but, in a few cases, produced nodular masses of acidophilic cells up to 50 μ in diameter. They could be seen with the unaided eye only when the process was so extensive as to render the serous surfaces translucent or opaque. Finally, routine examination of the thyroid gland during the later phase of the study has disclosed changes of uncertain nature. Again, the frequency recorded in table 2 may be too low. Areas of normal-appearing thyroid tissue are interspersed with irregular, apparently degenerating, acini containing little colloid and lined by small cells, irregular cords of small cuboidal cells, and circumscribed compact nodules up to 0.5 mm. in diameter. These nodules are made up of cords of larger cuboidal cells and distorted acini in which colloid can be seen (fig. 11). The changes are similar to those described by Schmidt (*7*) in thyroid tissue of mice receiving disrupted cells of the Ehrlich tumor during the first few hours of life.

A common feature among the mice examined was "ballooning" of nuclei and cellular enlargement affecting principally renal epithelium, bronchial, pulmonary alveolar, and thyroid cells like that described by Fisher *et al.* (*17*) for alveolar cells.

The changes observed in the inoculated mice were not all of a neoplastic nature. Mice injected with highly active preparations frequently became dwarfed. One-month-old mice that received fluid from control cultures usually weighed an average of 15 gm., while those receiving the tumor agent weighed an average of only 5 gm. The dwarfed mice remained small. Frequently, such mice developed an acute inflammatory process of the eyes (fig. 12), which resulted in an opacity of the cornea. The histological changes observed will be reported later.

Six Swiss mice that received the tumor agent developed severe anemias and a glomerular nephritis with anasarca. These mice did not have tumors, though litter mates which received some of the same material did. None of the controls have developed this lesion.

The results on titration of the tumor agent are given in table 3. Activity of #60 and #3607 sublines, from which dilutions were not recultured before inoculating into mice, could not be demonstrated beyond 10^{-3} and 10^{-4} dilutions, respectively. In the other two, #60 and #3469, in which the dilutions were recultured, tumors were induced in as high a dilution as 10^{-7}. Thus by reculturing, the amount of tumor agent was increased sufficiently to cause tumor induction at the higher dilutions. Mice that received the higher dilutions of the agent developed tumors at a later average age than those receiving the more concentrated material. No essential differences in the types of tumors induced in the mice that received the different dilutions were noted on gross examination.

Transmission of the agent, as evidenced by tumor induction, to the progenies of mice in which one or both parents had parotid-gland tumors has not been observed. Sixty offspring from (C3Hf $\times$ AKR)F_1 tumor-bearing mice were observed until time of death, an average period of 12 months, and none developed parotid-gland tumors. One female, however,

TABLE 3.—*Titration of tumor agent as determined by tumor induction in mice**

Subline	Passage	Tissue culture	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
60	1	Monkey-kidney	6/8	3/3	—	5/6	—	1/7	—	—	—
60	10	Mouse-embryo	—	9/12	—	13/15	—	15/18	—	2/13	0/12
3469	13	"	6/8	—	8/9	8/10	6/7	—	3/12	1/7	0/7
3607†	5	"	9/9	11/15	6/8	5/8	4/9	0/8	—	—	—
60†	8	"	3/7	—	6/8	7/8‡	—	—	—	—	—

*The denominator gives the mice surviving the inoculum and the numerator the mice with tumors.

†Dilutions were made from the passage material and inoculated into mice. In the others, the dilutions were inoculated into tissue culture and allowed to incubate 2 weeks before inoculating mice.

‡Dilution of 10^{-4} was not tested but mice inoculated with 0.33×10^{-3} did not develop neoplasms.

had a unilateral myoepithelioma of the salivary glands. Myoepitheliomas have been observed as spontaneous tumors in strains A and C mice (*18*) but this tumor has not been described in strains C3H or AKR or the F_1 hybrid of this cross. Similarly, 30 offspring from tumor-bearing Swiss mice have remained free of neoplasms in an observation period of 10 months.

A total of 420 Swiss mice and 200 (C3Hf $\times$ AKR)F_1 hybrid mice were inoculated when less than 24 hours old with supernatant fluids from mouse-embryo tissue-culture controls. Sixty of the control Swiss mice have died at 5 to 16 months of age. They were autopsied and the tissues examined histologically in the same manner as those of the mice that received the inoculated culture fluids. The only one with neoplasms was an 8-month-old male that developed a generalized leukemia. The others died of pneumonia or as a result of fighting.

Thirty hybrid mice have died at 6 to 20 months of age. Ten of these had a generalized leukemia which appeared at 9 to 20 months of age, and 2 females, 12 months old, had mammary tumors. The (C3Hf $\times$ AKR)F_1 hybrid mice normally have a 50 percent incidence of spontaneous leukemias which appear in mice over 9 months old (*4*). No other neoplasms have been observed in these mice.

None of the mice that received the control material developed parotid-gland tumors or any of the other tumors described. A total of 46 lots of mouse-embryo tissue cultures have been made; these represent the embryos from over 400 pregnant Swiss mice or a total of around 4,000 embryos. The supernatant fluids from the uninoculated cultures were passed at 2-week intervals in the same manner as the fluids from cultures carrying the tumor agent. Thus, 23 passages have been made with the control material and there was no evidence that a tumor-inducing agent had been picked up from any of the 4,000 embryos. It would appear that if the tumor agent, in a latent form, is widespread in a mouse colony, it is not passed *in utero*.

Discussion

In our earlier work it was reported that a tumor-inducing agent in cell-free extracts of leukemic mouse tissues produced a variety of neoplasms when injected into mice within 24 hours of birth (*2–4, 10, 15*). With these preparations the mice developed epithelial tumors, sarcomas, or early leukemias. Mice similarly inoculated with the tumor-inducing agent carried in monkey-kidney or mouse-embryo tissue cultures also developed multiple types of tumors, but none of the mice included in this report developed leukemia. This may be due to one of the following: 1) Mice with parotid-gland tumors frequently had atrophy of the thymus or developed an epithelial tumor of this organ. Many investigators consider the thymus as the site of origin for leukemias; if involved in another process it may be resistant to leukemia. 2) It is possible that if the mice had not developed parotid-gland tumors they would have lived longer and developed leukemias.

Whether we have one or more tumor agents is conjectural at this time. Multiplicity of effects produced by a single tumor agent in other species has been described by several investigators. The hemorrhagic lesion of chicks inoculated with Rous sarcoma virus as described by Duran-Reynals is a good example (*19*). It has been shown that only highly active preparations produce the hemorrhagic disease, and a curious type of liver lesion may also develop (*20*). Furth (*21*) found that a virus recovered from a fowl leukosis produced both neoplasms of the hematopoietic tissues and endotheliomas with giant-cell formation in many organs. Rose and Rose (*22*) described transformation of the Lucké tumor agent, which causes renal tumors in frogs, to an agent capable of eliciting chondrosarcomas by incorporating either in cells of young salamanders or in regenerating limbs of adult salamanders. The new agent when returned to frogs produced both renal and skeletal tumors. Recently, Carr (*23*) described a cystic papillomatous adenocarcinoma of the kidney in chicks inoculated, when less than 2 weeks old, with a fowl leukosis. Sarcomas also appeared at the site of inoculation.

The wider spectrum of neoplasms induced by the tumor agent carried in the mouse-embryo tissue cultures over those induced by the agent carried in the monkey-kidney cell cultures may be due to the following: 1) In the monkey kidney cultures the tumor agent was not carried beyond 4 passages because of simian viruses in the kidney cells as shown by the controls. These caused marked cytopathic changes which destroyed the sheets of cells. No evidence of contaminating viruses were observed in the control mouse embryo cultures; it was therefore possible to carry the tumor agent over many passages. 2) The cells of the monkey kidney cultures were primarily epithelial, whereas the mouse embryo cultures initially contained a variety of cell types and it is conceivable that the greater variety of cell types presented an opportunity for adaptation of the tumor agent. 3) Mouse embryo cells may simply present a medium better suited for the propagation of the tumor agent, and the greater the concentration of tumor agent inoculated into newborn mice the more tissues will be affected.

The multiple effects produced with leukemic cell-free extracts described in our earlier work were considered as due to one tumor agent. In our present work this still appears as the most likely explanation. Otherwise, one would have to postulate over 20 tumor-inducing agents in the tissue-culture fluids if one is to consider a specific agent for each primary tumor. It appears more reasonable at this time to consider the different cell types of newborn mice as being in a state receptive to the tumor agent. The agent may in time adapt to the specific type of cell.

It is conceivable that a tumor virus with many potentials may become adapted to one specific tissue only. By use of serologic and immunologic procedures, Beard (*24*) has found evidence of a relationship between the viruses of the avian leukemias, myeloblastosis, and erythroblastosis and he believes that these branch from the virus that induces avian lymphomatosis. Relationship to the Rous sarcoma virus by immunologic procedures was also shown.

Many of the neoplasms observed in the mice inoculated with the tumor agent were types that rarely occur spontaneously but may occasionally be induced by carcinogens in genetically susceptible hosts. With this highly potent tumor agent, however, genetic susceptibility appears to be of less significance since it was found to break not only certain strain barriers in the mice but also species barriers. For example, hamsters were found highly susceptible to tumor induction by this agent, both with nonfiltered tissue-culture fluid (*11*) and with fluid that had been filtered through bacteria-proof filters (*25*).

Extracts and filtrates of certain mouse tumors and fluids from tissue-culture preparations of such tumors have consistently produced more than one anatomical variety of neoplasms when injected into mice within 24 hours of birth. Perhaps just one agent as yet unidentified is responsible. The most reasonable hypothesis is that it is a virus. Work is under way to characterize this agent.

Summary

1) Five sublines of a tumor-inducing agent was passed serially, from 5 through 13 passages, in mouse-embryo tissue cultures with evidence of propagation of the agent.

2) A total of 23 different primary neoplastic lesions were produced in mice inoculated shortly after birth with the tumor agent.

3) Tissue-culture fluids retained their tumor-inducing activity after filtration through bacteria-proof filters.

4) The tumor agent was not specific for the strain of mice from which it was recovered.

5) Mice injected with supernatant fluids from uninoculated tissue cultures have remained free of unusual neoplasms.

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PLATES

463225—58——23

All photomicrographs are of sections stained with hematoxylin and eosin.

PLATE 120

FIGURE 1.—Hydronephrotic kidney from a (C3Hf × AKR)F_1 hybrid mouse. The parenchymal cells have been completely replaced by the sarcoma. × 2

FIGURE 2.—Mammary adenocarcinoma with a different architecture from the conventional murine carcinomas. Hair-follicle lesions overlying the mammary tumor. × 40

FIGURE 3.—Four-month-old (C3Hf × AKR)F_1 male with mammary adenocarcinoma in left axilla and left inguinal area. Mouse also has bilateral tumors of the parotid and sublingual glands and an epithelial thymoma.

FIGURE 4.—Tumor of a mucous gland in the trachea. Histologically it is the same as the pleomorphic tumors of the parotid glands. × 80

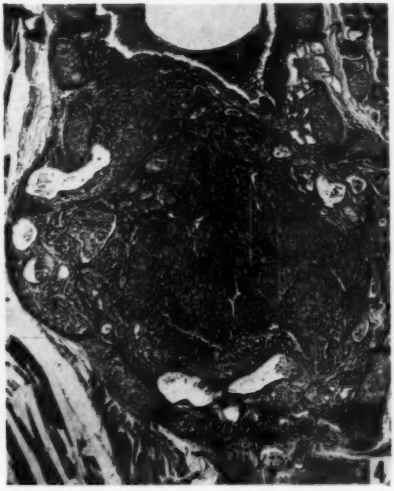


PLATE 121

FIGURE 5.—Three-month-old (C3Hf × AKR)F_1 female mouse with multiple epidermoid carcinomas of the hair follicles, multiple mammary tumors, and bilateral tumors of the parotid and submaxillary glands.

FIGURE 6.—Section through hair-follicle tumor showing areas of degeneration and atrophy and other areas of hyperplasia of the hair follicles. × 80

FIGURE 7.—Hair-follicle tumor metastasis to the lung. × 75

FIGURE 8.—Section through an adenocarcinoma of a sweat gland in a Swiss mouse. Sweat-gland tumors occurred bilaterally in the front paws. Anatomically they closely resemble the mammary tumors induced in these mice. ×130

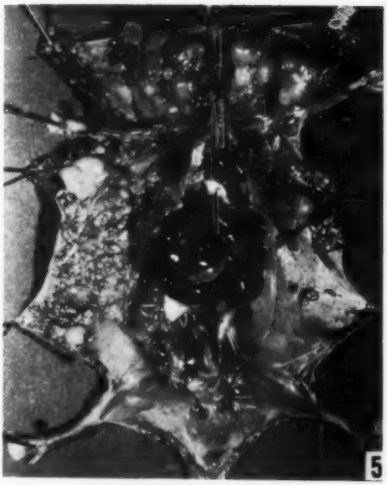


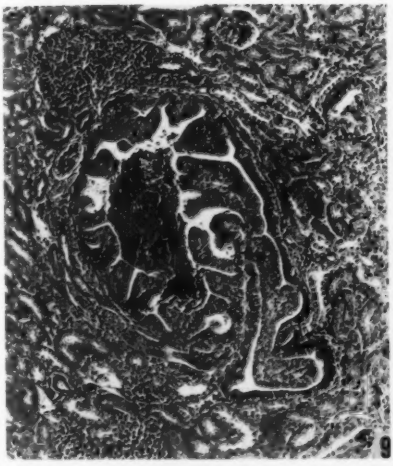
PLATE 122

FIGURE 9.—Papillary lesion of convoluted tubule in Swiss mouse with extreme dilatation of the tubule and pronounced papillary infolding of the epithelium into the lumen. × 143

FIGURE 10.—Papillary growth of the pleura in Swiss mouse characterized by finger-like projections of flattened cells continuous with those of the pleura. × 85

FIGURE 11.—Neoplastic nodule in thyroid of Swiss mouse showing hyperplasia of the acinar cells. × 280

FIGURE 12.—One-month-old Swiss mouse with acute inflammatory disease of both eyes, showing necrosis of cornea.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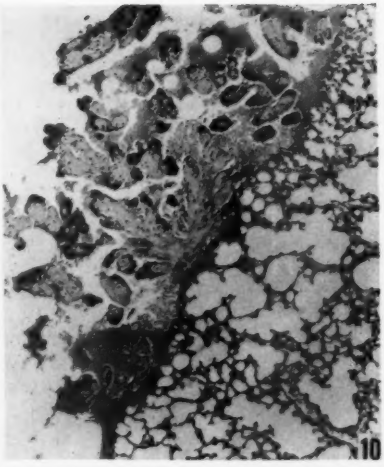
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Genetics of Experimentally Induced Erythroblastosis in Chickens [1]

NELSON F. WATERS, B. R. BURMESTER, *and* W. G. WALTER, *United States Department of Agriculture, Agricultural Research Service, Regional Poultry Research Laboratory, East Lansing, Michigan*

This report concerns a genetic study of the occurrence of erythroblastosis, artificially induced, in White Leghorn chickens. Erythroblastosis may be defined as a malignant neoplasm of the bone marrow; the cell involved is the erythroblast which proliferates intrasinusoidally, with little or no differentiation, throughout the marrow tissue. In its terminal stages it is manifested by a leukemic state and leukostasis, resulting in a generalized acute passive hyperemia and death. Strain RPL12 tumor preparation, which was used in this study (*1*, *2*), will cause, under certain conditions, a high incidence of erythroblastosis and a variable percentage of osteopetrosis (*3*). Strain RPL12 produces this disease after a moderately short but variable incubation period; the course is also quite short, with death usually occurring before 100 days of age. When the conditions are modified, this tumor strain also causes a high incidence of visceral lymphomatosis.

With certain unusual exceptions (*4*, *5*) the occurrence of erythroblastosis under natural conditions is quite rare, which indicates that it is not transmitted readily from chicken to chicken like lymphomatosis. The present experimental data on noninoculated and contact chickens are in accord with these field observations.

Materials and Methods

The chickens involved in these experiments were from 6 different inbred lines of single-comb White Leghorn chickens maintained at this laboratory for the past 18 years without the introduction of any outside stocks of chickens. The coefficient of inbreeding for the latest generation of these different inbred lines varied from 90.0 to 95.0 percent. The lines used had been selected routinely toward resistance or toward susceptibility to avian lymphomatosis (*6*, *7*).

A total of 970 female chickens obtained in 4 alternate weekly hatches from the 6 inbred lines was inoculated intraperitoneally at 1 day of age with 0.2 ml. of a preparation (L28) of strain RPL12. This inoculum was prepared by grinding in a blendor the leukotic liver of chickens of

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the 16th serial passage of strain RPL12 with 19 parts of a buffered salt solution and passing it through a porcelain filter candle. A total of 1,586 chickens from the same families as those used in the inoculated groups was hatched at alternate weeks of the same period, and the chicks were brooded and reared in different pens of the same house. These chickens served as noninoculated controls. An attempt was made to obtain from each individual dam-sire mating sufficient progeny to provide 10 female chicks for inoculation and 10 females for noninoculated controls.

All the chickens hatched in 1956 and inoculated at 1 day of age were placed in battery brooders for a 3-week period, and then transferred to brooder pens equipped with wire-paneled floors. The control chickens were reared in the same building under a similar general environment but were not in direct contact with the inoculated chickens.

Chicks hatched in 1957 were inoculated *via* the cubital vein at 14 days of age with 0.2 ml. of a filtrate (L31) prepared in the same manner as the inoculum (L28) used the previous year. Prior tests indicated that inoculum L31 was more potent than L28; therefore it was diluted 1:3 just before its use.

Like the procedure in 1956, except that in 1957 both males and females were used, a sample of 1,202 chickens from all inbred lines was inoculated. Noncontact control chickens totaling 1,728, representing all inbred lines, were available. In addition, 586 chickens were in direct contact with the inoculated chickens. The inoculated and noninoculated contact-control chickens had access to static or non-running water during the entire 100-day period of the experiment.

Experimental Results

The percentage mortality from erythroblastosis in both the inoculated and noninoculated control groups, for lines of chickens hatched in 1956 and 1957, is shown in table 1, and in table 2 the frequency of death in days following inoculation.

Death from erythroblastosis among both the 1956 and 1957 populations occurred with few exceptions before 100 days of age. These distributions of age-at-death are in accord with the known behavior of similar groups of chickens when a preparation of strain RPL12 is used as the inoculum (*3*). The average age-at-death following inoculation for the chickens of the 1956 population was 45.2 days and for the 1957 population, 38.9 days. Thus the chickens receiving the intravenous inoculation at 14 days of age died on an average of 6.3 days earlier than those receiving the intraperitoneal inoculation at 1 day of age. It is doubtful that this difference in days at death between the two different populations has any important implication.

Only one case of erythroblastosis was recorded among the 586 contact-control birds of the 1957 population. Another case occurred among the 1,728 controls of the 1957 population and none in the 1956 control population. The possibility that these two recorded cases were errors in diagnoses should be considered since tissues of only a few of the birds were

Vol. 20, No. 6, June 1958
463225—58——24

TABLE 1.—*Percentage mortality to 100 days post inoculation from erythroblastosis and osteopetrosis among different inbred lines of chickens, both inoculated and noninoculated, for 1956 and 1957*

1956

Line	Number chickens	Inoculated at 14 days of age				Number chickens	Controls			
		Erythroblastosis		Osteopetrosis			Erythroblastosis		Osteopetrosis	
		No.	%	No.	%		No.	%	No.	%
6	153	1	0. 6	1	0. 6	218	0	0. 0	0	0. 0
7	199	1	1. 0	0	0. 0	229	0	0. 0	0	0. 0
9	217	25	11. 5	2	0. 9	276	0	0. 0	0	0. 0
10	27	5	18. 5	0	0. 0	311	0	0. 0	0	0. 0
14	38	11	28. 9	0	0. 0	223	0	0. 0	0	0. 0
15	336	82	24. 4	18	5. 4	329	0	0. 0	0	0. 0
Total	970	125	12. 9	21	2. 2	1586	0	0. 0	0	0. 0

1957

Line	Number chickens	Inoc. at 14 days of age				Number chickens	Controls				Number chickens	Contact controls			
		No.	%	No.	%		No.	%	No.	%		No	%	No.	%
6	204	9	4. 4	2	1. 0	316	0	0. 0	0	0. 0	145	0	0. 0	0	0. 0
7	214	3	1. 4	0	0. 0	311	1	0. 3	0	0. 0	71	0	0. 0	0	0. 0
9	227	19	8. 4	2	0. 9	334	0	0. 0	0	0. 0	234	1	0. 4	0	0. 0
10	228	56	24. 6	0	0. 0	282	0	0. 0	0	0. 0	14	0	0. 0	0	0. 0
14	147	77	52. 4	5	3. 4	188	0	0. 0	0	0. 0					
15	182	49	26. 9	6	3. 3	297	0	0. 0	0	0. 0	122	0	0. 0	0	0. 0
Total	1202	213	17. 7	15	1. 2	1728	1	0. 1	0	0. 0	586	1	0. 2	0	0. 0

TABLE 2.—*Frequency distribution for postinoculation death from erythroblastosis for 6 different inbred lines of chickens*

Line	Postinoculation death (*days*):									
	1–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
1956										
6							1			
7								1		
9			2	6	9	4	1	1		2
10				1	2	1	1			
14			3	4	3	1				
15			8	25	32	10	6		1	
1957										
6					4	1			2	2
7				1	1		1			
9			5	10	3	1				
10			9	20	19	5	1	2		
14		1	16	32	16	8	2	2		
15		6	25	10	4	2	1		1	

examined microscopically. In general, diagnoses were based on gross alterations found at necropsy. Such alterations and others obtained after inoculation of chickens with strain RPL12 have been extensively studied in many different experiments (*2*, *3*); therefore microscopic examination of all birds that died in this experiment was considered unnecessary. It is quite apparent that erythroblastosis, although readily transmitted by inoculation, is not readily, if at all, transmitted by close contact with inoculated chickens.

Marked differences in mortality from erythroblastosis among the inoculated chickens occurred between inbred lines. The low incidence of erythroblastosis that occurred in lines 6 and 7, in both 1956 and 1957, indicates a high resistance to this disease. Lines 9, 10, 14, and 15 show a much higher incidence of erythroblastosis for both years. The number of chickens involved in lines 10 and 14 for 1956 are too few for a reliable estimate. However, with larger numbers of chickens from these 2 lines used in 1957, the degree of susceptibility to erythroblastosis is substantiated. Clearly there are pronounced genetic differences in resistance to erythroblastosis among the chickens of the 6 different inoculated inbred lines.

It would appear, also, that the inoculum used on the various lines of chickens influenced the incidence of osteopetrosis, table 1. Cases of osteopetrosis have occurred in both the 1956 and 1957 inoculated chickens, while none occurred in the noninoculated control groups for either year. With few exceptions, the osteopetrotic cases, shown in table 1, were in combination with erythroblastosis. The limited appearance of osteopetrosis in the inoculated chickens does not warrant a genetic interpretation at this time.

A further analysis of individual lines and matings within lines adds considerable credence to a genetic interpretation of resistance to the erythroblastic form of fowl leukosis among inoculated chickens.

Data from 4 different matings in 1956 from inbred line 15 are shown in table 3. Each mating consisted of a single male and a number of females. Thus 4 different males were mated to 4 different sets of females. All the males and females had a high relationship to each other, with a coefficient of inbreeding in excess of 90.0 percent. Progeny from each male was challenged with a standard dose of inoculum as previously described. A noninoculated group of control progeny from each family and from each different mating was available. The progeny obtained from matings 1, 2, and 3 in line 15 showed a fairly high average mortality from erythroblastosis. The progeny from mating 4, line 15, showed no erythroblastosis. No evidence of the disease occurred in the noninoculated control matings. Because this lack of mortality in mating 4 appeared unusual, a repetition of mating 4, together with mating 3, which showed 38.5 percent mortality from erythroblastosis, was made several months later. Results obtained with the progeny of these 2 repeat matings (3 and 4) are also shown in table 3. The mortality is comparable to that obtained in the first matings.

TABLE 3.—*Percentage mortality to 100 days post inoculation, from erythroblastosis and osteopetrosis, for different matings within inbred line 15, inoculated chickens, for 1956**

Mating	Male	Number of progeny	Erythroblastosis		Osteopetrosis	
			Number	Percent	Number	Percent
1	Q729T	82	25	30. 5	9	10. 9
2	Q790R	63	20	31. 7	5	7. 9
3	Q760K	96	37	38. 5	4	4. 2
4	Q760B2	95	0	0. 0	0	0. 0
	Total.....	336	82	24. 4	18	5. 3
Repeat mating from above						
3	Q760K	51	22	43. 1	7	13. 7
4	Q760B2	75	0	0. 0	0	0. 0
Males exchanged in 3 and 4						
3A	Q760B2	46	0	0. 0	0	0. 0
4A	Q760K	75	27	36. 0	5	5. 6

*Two matings are repeated with the same male and with males exchanged. All inoculations made intraperitoneally at 1 day of age.

The next logical step was the reciprocal exchange of males between matings 3 and 4 of line 15, and the inoculation of additional groups of progeny. The response of the progeny is given in table 3 under matings 3A and 4A. The females of mating 3, when previously mated to sire Q760K,

in 2 trials produced progeny that showed high susceptibility to the development of erythroblastosis; however, when the sire was changed to Q760B2 (mating 3B), the resulting progeny showed complete resistance. In addition, other females, when mated in the first 2 tests with Q760B2 (mating 4), produced progeny, none of which developed erythroblastosis, but when the same females were mated to Q760K (mating 4B), a high incidence of the disease developed in the progeny.

It is clear that male Q760B2, which was used in matings 4 and 3A and produced a total of 206 progeny when mated with 22 females, was capable of transmitting to its progeny complete resistance to the development of erythroblastosis. It is equally clear that another male, Q760K, when mated to the same females, transmitted genes capable of expressing susceptibility to erythroblastosis. The 2 males that gave these divergent responses were full brothers. These differences in the performance of the progeny of the 2 brothers are of particular interest in view of the fact that the average inbreeding coefficient of this line exceeds 90.0 percent.

The adequacy of the exposure of the chickens to the virus causing erythroblastosis may be judged by the result that 38.7 percent of the 222 progeny of male Q760K died of erythroblastosis.

The results obtained with the individual male matings of line 15 during 1956 were like those obtained with 3 matings from line 9 for the same year. Mortality after inoculation with strain RPL12 of the progeny from these matings is shown in table 4. Progeny of mating 1 developed no erythroblastosis; whereas, matings 2 and 3 both produced progeny, some of which developed the disease. A repeat mating with the same sire and dams used in matings 1 and 3 produced similar results. Unfortunately, the male in mating 1 died before a reciprocal exchange of males was realized.

In both line 9 and line 15 matings for 1956, there were also differences in the occurrence of osteopetrosis. Osteopetrosis occurred only in the progeny of matings that also developed erythroblastosis. However, the

TABLE 4.—*Percent mortality to 100 days post inoculation, from erythroblastosis and osteopetrosis, for different matings within inbred line 9, inoculated chickens, for 1956**

Mating	Male	Number of progeny	Erythroblastosis		Osteopetrosis	
			Number	Percent	Number	Percent
1	Q415L	76	0	0.0	0	0.0
2	Q447K	81	7	8.6	0	0.0
3	Q419J	60	20	33.3	2	3.3
Total		217	27	12.4	2	0.9
Repeat mating from above						
1	Q415L	68	0	0.0	0	0.0
3	Q419J	35	7	20.0	1	2.8

*All inoculations made intraperitoneally at 1 day of age.

assumption is not warranted that the susceptibilities to each of these two diseases are related, inasmuch as 7 of the 21 cases occurring in the 1956 inoculated population were not accompanied by evidence of osteopetrosis. No cases of osteopetrosis appeared in any of the noninoculated control chickens.

When matings were organized for the year 1957, males and females were selected from the noninoculated control sibs from the same families in lines 9 and 15, which were hatched during 1956 and which had shown either a high or low incidence of erythroblastosis after inoculation. The percentage mortality from erythroblastosis and osteopetrosis among the 4 matings from each of lines 9 and 15 is presented in table 5. One mating of line 9 produced 59 progeny of which 32.2 percent died of erythroblastosis. Three matings of line 9 produced 168 progeny, none of which developed erythroblastosis. In line 15 the progeny of 3 matings, each sired by a different male, showed a relatively high mortality from erythroblastosis, and the progeny of a fourth mating showed complete resistance to the disease. Deaths from osteopetrosis were relatively few in both line 9 and line 15 progeny.

TABLE 5.—*Percentage mortality to 100 days post inoculation, from erythroblastosis and osteopetrosis, for different matings within inbred lines 9 and 15, both inoculated and noninoculated, during 1957*

Mating	Male	Inoculated					Controls				
		Number	Erythroblastosis		Osteopetrosis		Number	Erythroblastosis		Osteopetrosis	
			No.	%	No.	%		No.	%	No.	%
Line 9											
1	R409C	57	0	0.0	0	0.0	118	0	0.0	0	0.0
2	R430F	59	19	32.2	1	1.7	149	0	0.0	0	0.0
3	R466G	58	0	0.0	0	0.0	107	0	0.0	0	0.0
4	R424G	53	0	0.0	0	0.0	106	1	0.9	0	0.0
Total		227	19	8.4	1	0.4	480	1	0.2	0	0.0
Line 15											
1	R705D	44	25	56.8	0	0.0	70	0	0.0	0	0.0
2	R786D	51	0	0.0	0	0.0	92	0	0.0	0	0.0
3	R746D	49	9	18.4	3	6.1	101	0	0.0	0	0.0
4	R759A	38	15	39.4	5	13.1	35	0	0.0	0	0.0
Total		182	49	26.9	8	4.4	298	0	0.0	0	0.0

A summary of the ratio of chickens that died from erythroblastosis to the total number of progeny produced from each single sire and dam, by inbred line and by years, is shown in tables 6 (1956) and 7 (1957). Altogether, for both years, 13 sires of the 37 used, when mated to 107

TABLE 6.—*Ratio of chickens dead from erythroblastosis to total number of progeny produced from single sires and dams by inbred lines for the year 1956*

Dams	Line 6 (*dead/total*)			Line 7 (*dead/total*)			Line 9 (*dead/total*)			Line 15 (*dead/total*)			
	Sire:			Sire:			Sire:			Sire:			
	1	2	3	1	2	3	1	2	3	1	2	3	4
1	0/4	0/12	0/5	0/3	0/1	0/6	0/4	0/11	0/11	1/6	0/3	1/3	0/5
2	0/4	0/1	0/6	0/1	0/2	0/11	0/9	1/9	2/6	2/9	1/6	1/1	0/11
3	0/6	0/4	0/2	0/4	0/8	0/6	0/14	5/11	2/9	2/3	4/12	2/7	0/7
4	0/9	0/3	0/3	0/3	0/10	0/9	0/8	1/7	0/1	1/7	1/4	4/8	0/1
5	0/5	0/6	0/3	0/4	0/5	0/6	0/10	0/10	1/2	2/7	2/7	5/11	0/9
6	0/4	0/7	0/2	0/7	1/7	0/6	0/7	0/6	1/2	2/6	2/9	1/8	0/11
7	0/2	0/7	0/7	0/7	0/9	0/1	0/9	0/7	5/10	4/10	2/6	6/10	0/5
8	0/1	0/1	0/7	0/6	0/9		0/12	0/4	1/9	2/6	2/4	3/12	0/9
9	1/3		0/5	0/10	0/4		0/3	0/1	6/10	3/4	5/11	3/6	0/1
10	0/2		0/6	0/2	0/9			0/10		0/2	1/1	2/7	0/12
11	0/6		0/2	0/1	0/14			0/5		0/9		2/5	0/2
12	0/11		0/1	0/3	0/8					5/8		6/11	0/10
13	0/1		0/3		0/8					1/5		1/7	0/6
14	0/2				0/9								0/1
15													0/4
Total	1/60	0/41	0/52	0/51	1/103	0/45	0/76	7/81	18/60	25/82	20/63	37/96	0/95

TABLE 7.— *Ratio of chickens dead from erythroblastosis to total number of progeny produced from single sires and dams by inbred lines for the year 1957*

Dams	Line 6 (*dead/total*) Sire:				Line 7 (*dead/total*) Sire:				Line 9 (*dead/total*) Sire:				Line 10 (*dead/total*) Sire:				Line 14 (*dead/total*) Sire:				Line 15 (*dead/total*) Sire:			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	1/10	0/6	0/6	3/9	1/8	0/9	1/10	0/10	0/11	0/10	0/11	0/9	0/1	1/5	0/7	0/8	0/1	2/2	0/3	1/3	6/9	0/7	5/10	1/3
2	0/8	0/8	3/7	0/9	0/9	0/8	0/9	0/9	0/10	6/10	0/9	0/9	0/3	1/4	0/3	0/5	3/4	1/1	8/9	4/5	1/4	0/9	0/9	6/8
3	0/10	0/7	0/9	0/6	1/10	0/10	0/10	0/10	0/9	1/10	0/7	0/6	1/3	0/1	6/9	0/1	1/2	0/3	1/1	3/11	5/6	0/8	0/7	1/6
4	0/8	0/9	0/6	1/9	0/7	0/9	0/5	0/5	0/7	6/8	0/9	0/10	1/3	1/1	1/3	0/1	6/6	0/3	1/1	0/3	5/9	0/8	3/4	4/8
5	0/9	0/6	0/10	0/7	0/10	0/10	0/10	0/10	0/10	2/11	0/10	0/9	4/6	1/3	2/9	1/5	1/1	1/1	3/9	7/10	4/8	0/9	0/10	2/6
6	0/10	0/10		1/7	0/10	0/8	0/10	0/10	0/10	4/10	0/12	0/10	0/7	5/9	1/3	0/3	1/1	1/1	1/4	1/1	4/8	0/10	1/9	1/7
7		0/8		0/10	0/6									4/9	2/7	0/3		2/2	1/2	1/4				
8														1/9	0/6	0/2			2/2	1/6				
9														0/7	0/7	1/2			4/4	2/3				
10														1/4	2/3	1/1				0/1				
11														0/3	1/3	0/4				4/7				
12														2/8	1/6	1/1				1/9				
13														3/4	3/3	0/4				0/1				
14														0/1	0/2	2/2				10/12				
15														1/4	2/11	0/2				2/8				
15														2/7										
16																								
Total	1/55	0/54	3/38	5/57	2/60	0/54	1/54	0/46	0/57	19/59	0/58	0/53	6/23	23/79	21/82	6/44	12/15	7/13	21/35	37/84	25/44	0/51	9/49	15/38

different dams, produced 733 progeny showing no evidence of erythroblastosis. The remaining 24 sires all produced 1 or more progeny that developed erythroblastosis. Among the 331 dams used during 1956 and 1957, 198 produced a total of 1,292 progeny showing no cases of erythroblastosis. The remaining 133 dams all produced 1 or more progeny, in each individual family, showing the disease.

The data in tables 6 and 7 show that 68 dams each produced 5 or more progeny that remained free from erythroblastosis, even though the sires with which they were mated did produce affected progeny when mated to other dams. This suggests that the female parent can also influence a genetic expression of resistance to erythroblastosis in the progeny.

Discussion

Genetic resistance and susceptibility of a host to a pathogen have been demonstrated repeatedly in many animals. In most instances it has been possible to demonstrate only relative degrees of resistance and the mode of inheritance is problematical. However, Sabin (*8*) demonstrated the complete resistance in albino mice, of the Princeton Rockefeller Institute, to the 17D strain of yellow-fever virus, thus providing an example of precise genetic response in host-pathogen relationship. Sabin demonstrated that PRI albino mice may show 100 percent resistance to the yellow-fever virus, whereas Swiss mice are 100 percent susceptible. Crosses between the PRI albino mice and Swiss mice suggest that resistance to 17D strain yellow-fever virus is dependent upon a single autosomal dominant factor for expression.

Evidence that resistance to the mammary-tumor agent in mice is dependent upon a relatively simple genetic mechanism is presented by Heston *et al.* (*9*). Strain C3H female mice susceptible to mammary-tumor agent were crossed with males from strain C57BL mice resistant to mammary-tumor agent. The resultant female progeny from this strain cross of C3H females with C57BL males were in turn backcrossed to strain C57BL males through 7 backcross generations. By the third backcross generation the tumor agent had been eliminated by this concentration of the genes of strain C57BL. Heston and his coworkers suggest that these 2 strains of mice differ by only a few genes which control the "propagation and transmission of the mammary-tumor agent," or possibly, by only a single pair of genes.

The evidence presented on resistance to virus-induced erythroblastosis in this report suggests a rather clear-cut genetic pattern. The ability of individual males and females to transmit to their progeny complete resistance to the development of erythroblastosis, an infectious neoplastic disease, is unique in studies involving resistance and susceptibility of a host to a specific disease.

Rarely do chickens of the inbred lines maintained at this laboratory die from naturally occurring erythroblastosis. If, however, the chickens of these same inbred lines are challenged with the virus causing this disease at a young age, they will live or die according to their genetic

capabilities. An analysis of mortality from virus-induced erythroblastosis within the progeny of individual sires and dams provides evidence for genetic segregation of genes responsible for resistance and susceptibility.

The data presented show that certain males have transmitted to their progeny complete resistance to erythroblastosis, even though some of the females with which they are mated can be considered genetic carriers of susceptible genes. It is equally clear that a substantial number of females can likewise transmit complete genetic resistance to their progeny. An interpretation of the results on the genetic behavior of many sires and dams to induced erythroblastosis permits the hypothesis that resistance to this disease involves a relatively simple type of inheritance possibly dependent for complete expression on only one pair of autosomal dominant genes. Final evidence that a single pair of dominant genes control resistance to induced erythroblastosis must await further testing. In many matings involving erythroblastosis, wherein a single male is mated to several dams, the ratios of infected to noninfected progeny are highly suggestive of segregation based on a single gene difference. It is difficult with the present data to isolate, because of too few progeny in a family and mixed genotypes, males and females both of which are homozygous recessive. The data do show that such homozygous recessive males and females may exist in line 14 (table 7).

Summary

This report concerns a genetic study of the occurrence of erythroblastosis, artificially induced, in 6 different inbred lines of White Leghorn chickens. The inbred chickens have been maintained at this laboratory for the past 18 years without the introduction of any outside stocks of chickens. The coefficient of inbreeding for the latest generation of these different inbred lines varied from 90.0 to 95.0 percent.

Altogether, 1,972 inoculated chickens were involved and these represented the combined progeny of 37 sires and 335 dams. Marked differences in mortality from erythroblastosis existed among the inoculated chickens of the different lines. Progeny from 2 lines, 6 and 7, of the 6 inbred lines were nearly 100 percent resistant to erythroblastosis. The data presented show that 13 males, of 37 used, when mated to 107 different dams were capable of transmitting complete resistance to 733 progeny. Similarly, 68 dams, each producing 5 or more progeny, transmitted complete resistance to their progeny, even though the sires with which they were mated did produce affected progeny when mated to other dams.

An interpretation of the results on the breeding behavior of many sires and dams permits the hypothesis that resistance to erythroblastosis involves a relatively simple type of inheritance, possibly dependent for complete expression on only one pair of autosomal dominant genes.

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Studies on Transplantable Testicular Teratomas of Strain 129 Mice [1,2]

LEROY C. STEVENS, *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

The testicular teratomas that develop congenitally in about 1 percent of inbred strain 129 male mice are chaotic mixtures of many tissue types of various degrees of maturity (*1*, *2*). Grafts of these tumors took and survived indefinitely, but they grew progressively in only 4 of 19 cases observed. Strain 129 teratomas have been maintained as transplantable tumors in order to investigate their developmental capacities over extended periods of time and to observe changes, particularly in morphology, that might occur during many transplant generations. As Snell (*3*) has pointed out, it is an almost universal characteristic of transplantable tumors that any of several of their biological properties may change during the course of repeated transfers. These properties include growth rate, degree of cellular differentiation, host specificity, dependence upon hormonal stimulation, and chromosome number.

Changes in the behavior of mammalian tissue cells after extended periods of cultivation *in vitro* have been reported by several workers. Lines have been established from cells which were derived from a single cell of normal subcutaneous adipose tissue and which differ in their ability to produce sarcomas when injected into mice (*4*). Differences in histocompatibility and the morphology of these cells were also demonstrated.

Jackson and Brues (*5*) recognized the advantage of using transplantable teratomas in investigations of changes during serial transfer of tumors and in studies of the nature of the stem cell of teratomas. They found that during the course of serial transplantation, a murine ovarian embryoma showed no essential change. Random selection of tissue for transplantation did not result in sorting out tissue types. All grafts were found to be composed of undifferentiated "embryonal" cells and several kinds of tissues. The embryonal cells contained virtually all of the mitotic figures, and it was suggested that some of these cells matured while others were responsible for the growth of the tumor. Fekete and Ferrigno (*6*) observed a transplantable mouse ovarian teratoma for 9 transplant genera-

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tions and noted that all tumors, like the original teratoma, were mixed, containing undifferentiated embryonal cells and a variety of differentiated tissues. An attempt to isolate different components of the tumor by transplanting small pieces that were pigmented or soft white parts was not successful. Currently this tumor (E6496) is in the 54th transplant generation, and morphologically different sublines have arisen (Fekete, personal communication).

It is the purpose of this paper to described some transplantable strain 129 testicular teratomas and the morphological changes they have undergone during prolonged serial transfer. These pleomorphic tumors are particularly suitable for the detection of progression along morphological lines and for observations of changes preceding irreversible transformations. Most of the emphasis is placed on a study of multiple lines derived from a single primary testicular teratoma.

Materials and Methods

All the transplantable testicular teratomas to be described were derived from primary tumors of inbred strain 129 mice, and, for the most part, this paper deals with sublines derived from a primary tumor found in a male approximately 60 days old. Solid and cystic parts of the tumors were minced together and grafted subcutaneously by trocar into the right flank of male or female strain 129 mice 4 to 6 weeks of age. Some tumors were homogenized in a 0.85 percent NaCl solution and the resulting cell suspensions were injected subcutaneously or intraperitoneally. In order to prevent contamination of one tumor by another, all instruments were boiled for several minutes before being used. Over 1,400 grafts were observed and portions of many of these were placed in Vandegrift's fixative, sectioned, and stained with hematoxylin and eosin for microscopic examination.

Observations

Primary Teratomas Providing Grafts

The primary teratomas providing material for grafts varied in their composition, though most of those taken from donors 20 days of age and older contained neural tissue, notochord, epithelium, cartilage, bone, and muscle. In table 1 the kinds of tissues observed in sections of some of these tumors are presented. Embryonal cells were observed in only 6 of the 15 tumors examined histologically. Two of these (395 and 396) were from animals less than 16 days of age when all primary strain 129 teratomas contain embryonal cells.

Portions of 19 primary teratomas were grafted into 116 hosts. The donors ranged in age from 10 to 210 days, and the hosts from less than 1 day to 94 days (table 2). Samples of 2 of the 4 tumors that yielded progressively growing grafts had undifferentiated embryonal cells and immature neuroepithelium. The composition of the *grafts* from these tumors is, of course, not known. In some cases most of the tumor was transplanted, which left only a small fragment for microscopic examination,

TABLE 1.—*Tissue types found in portions of the primary teratomas providing grafts. Identification numbers of tumors that yielded progressively growing grafts are italicized*

Identification No.	Age of mice (*days*)	Simple epithelium	Ciliated epithelium	Stratified squamous epithelium	Goblet cells	Epidermis	Notochord	Fat	Cartilage	Bone	Marrow	Muscle	Immature neural tissue	Neuroglia	Large neurons	Undifferentiated embryonal cells
215	210	x	x											x		
233	44	x	x	x	x		x	x	x	x	x	x		x		
277	Adult	x	x	x	x			x	x	x	x	x		x		
289	60	x	x	x	x		x		x	x	x			x	x	
305	18	x	x	x					x			x	x	x		
324	22	x	x		x		x		x	x	x	x	x	x	x	x
332	31	x	x											x		
369	26	x	x	x	x		x	x		x		x		x	x	
395	15	x	x				x						x	x		x
396	10	x	x				x						x	x		x
402	60	x	x	x	x			x	x	x	x	x		x	x	
425	24	x	x		x		x		x	x	x	x		x	x	
490	30	x	x	x	x		x	x	x	x	x	x	x	x	x	x
530	Adult	x	x	x	x	x	x	x	x	x		x	x	x		x
1062	25	x	x	x	x	x		x	x	x		x	x	x		x

so that the significance of these data in attempting to correlate the composition of the graft with its ability to grow progressively is limited.

First-Generation Grafts

Eighty-two of the 116 first-generation grafts were recovered (*see* table 2). Fourteen of these grew progressively, while the others survived many months as nodules composed of several kinds of differentiated, nonproliferating tissues. The progressively growing grafts were derived from 4 primary teratomas, each of which provided grafts that grew progressively in more than one host. Our data give no indication that the growth and differentiation of tissues in the grafts was markedly influenced by the site of the graft (whether intraperitoneal or subcutaneous), the preparation of the graft (minced or homogenized), the age of the donor or host, or the sex of the host.

The composition of the first-generation grafts at the time of autopsy of the hosts varied considerably. Those that grew progressively were composed purely of embryonal cells (233), mainly of undifferentiated cells but containing several differentiated tissues (1062), or principally of differentiated tissues (402 and 530). Each kind of tissue occurring in the spontaneous tumors was represented in these grafts, including various kinds of epithelia, nervous tissue, cartilage, bone, marrow, glandular structures, muscle, notochord, and adipose tissue.

The first-generation grafts that did not grow progressively were devoid of embryonal cells and were composed entirely of adult-type tissues (fig. 1). Portions of 12 of these growths were retransplanted; in all cases they

TABLE 2.—*Characteristics of donors, hosts, and grafts of strain 129 teratomas*

Identification No.	Number of grafts	Age of host (*days*)	Preparation of graft	Site of graft	Grafts found at autopsy		Number of grafts that grew progressively
					Number	Age (*days*)	
215	6	±30	Minced	Subcutaneous	5	129–369	0
233	6	94	"	"	5	20	4
277	6	±30	"	"	4	75–135	0
289	3	±30	"	"	0	72	0
305	3	±30	"	"	1	120	0
332	3	91	"	"	3	365	0
324	3	±30	"	"	3	365	0
369	5	±30	"	"	4	403	0
391	4	30	"	"	4	365	0
395	1	±30	"	"	1	75	0
396	1	45	"	"	1	420	0
402	18	25–59	"	"	18	120–550	2
	5	25–59	Homogenized	Intraperitoneal	1	365	0
425	2	25	Minced	Subcutaneous	1	403	0
471	1	Less than 1	Homogenized	"	0	375	0
472	8	"	"	"	0	120	0
490	3	56	"	Uterine lumen	0	150	0
	2	56	"	Subcutaneous	0	270	0
530	20	30	Minced	"	17	123–434	0
	5	30	Homogenized	"	4	64–221	1
	5	30	"	Intraperitoneal	4	90–150	3
1307	2	10	Minced	Subcutaneous	2	150	0
1062	4	30	"	"	4	28–46	4
Total.....	116	.....	.....	.....	82	.....	14

failed to grow progressively but survived as nodules composed of several tissues types.

Composition and Behavior of Progressively Growing Transplantable Teratomas

The major emphasis in this paper is on descriptions of sublines of a transplantable teratoma referred to as 402. The characteristics of 3 other transplantable strain 129 teratomas will be mentioned briefly for purposes of comparison. The primary tumors from which these were derived were designated 233, 530, and 1062, and the transplantable tumors derived from them were given corresponding designations.

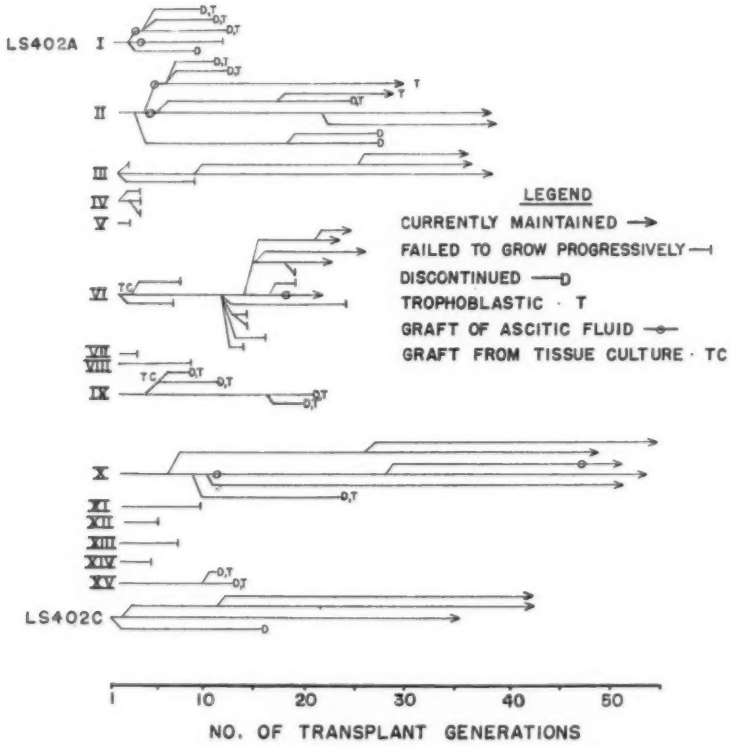
The primary tumor 233 was found in the testis of a 44-day-old mouse and the sample examined histologically was well-differentiated tissue. Parts of this tumor were minced and grafted subcutaneously to 6 hosts. Twenty days later, one of the grafts measured approximately 2 cm. in diameter and was retransplanted. Microscopic examination of the first-generation transplant revealed that it was composed entirely of embryonal cells, and throughout 76 subsequent passages we have observed no morphological changes. The central portions of transplantable tumor 233 were hemorrhagic and necrotic.

The primary teratoma designated 530 was grafted from the left testis of an adult mouse to 30 recipients. Twenty mice received minced subcutaneous grafts *via* trocar. All these grafts survived and, after 4 to 15 months, were harvested. Sections of the 2 largest growths (approximately 1.5 cm. in diameter) were examined. Both contained many types of differentiated tissues and, in addition, 1 had embryonic cells and immature tissues. These 2 were retransplanted but neither grew progressively. Ten animals were injected with homogenized primary tumor, 5 subcutaneously and 5 intraperitoneally. One of the subcutaneous and 3 of the intraperitoneal grafts grew progressively, and all were composed entirely of cells resembling mouse trophoblast, described later. The intraperitoneal grafts formed solid nodules attached to the peritoneum, free-floating single cells, and cell clusters. These tumors were propagated for 9 transplant generations. No changes occurred, and the tumors were discarded since similar growths resulted from grafts of sublines of 402.

Teratoma 1062 was found in the left testis of a 25-day-old mouse. This tumor contained differentiated tissues but was composed mainly of immature neural tissue and embryonic cells. All 4 grafts from this primary tumor grew progressively and after 28 to 46 days were retransplanted. Four sublines of the tumor were carried for 10 transplant generations. Samples from all descendant growths were like the tumor from which they were derived in that they produced small regions of differentiated tissues, but immature neural tissue and embryonic cells predominated.

Teratoma 402 was taken from the left testis of a 60-day-old male. It was composed of many differentiated tissue types, and in the portion prepared for microscopic examination there were only a few small patches of embryonal cells. About half of this tumor was minced and trans-

planted subcutaneously into 18 hosts. Five other animals received intraperitoneal injections of homogenized tumor. All the minced grafts were harvested, but remnants of only 1 of the homogenized grafts were recovered. Two subcutaneous grafts grew progressively and were designated 402A and 402C. Another (402B) was retransplanted to 5 hosts but the grafts did not grow progressively and microscopic examination of the donor sample revealed no embryonic tissue. The relationships between the sublines of 402A and 402C are represented in text-figure 1.



TEXT-FIGURE 1.—Diagrammatic representation of passages of tumor sublines derived from primary testicular teratoma 402. Several of these tumors are being maintained currently (——→), some became differentiated and failed to grow progressively (——|), some were discontinued (——D), some resembled mouse trophoblast (T), some were homogenized, inoculated intraperitoneally, and the resulting ascitic fluid grafted (–⊖–), and some were grafts of tumor that had been cultivated *in vitro* (TC).

Transplantable Sublines of Testicular Teratoma 402

Portions of 402A were transplanted into 15 hosts (designated I to XV in text-fig. 1). All of these grafts grew and each was retransplanted. The first to reach a transplantable size served as the progenitor of a subline designated 402A I. The remaining grafts were progenitors of sublines

II to XV. The composition of the second-generation grafts of sublines 402A I to XV at the time of autopsy of the hosts was, in general, similar. In all, embryonal cells and immature neuroepithelium were the most conspicuous components, though they all had various types of epithelia as well. There was skeletal muscle in 12 of the 15 tumors, cartilage in 4, and bone in 2.

In the course of subsequent transplantation, morphological differences arose among some of the sublines derived from testicular teratoma 402. Some of these differences have persisted through 24 to 54 transplant generations. The sublines can be classified into 6 different categories with characteristic patterns of differentiation. An additional category is listed since it has been observed in another transplantable teratoma (233).

It should be pointed out that some of the categories in the following classification of transplantable strain 129 teratomas are more or less arbitrary and some tumors have characteristics in-between 2 categories. Furthermore, the classification is based upon the characteristics of these tumors at present and further changes in some of them may be expected as they have occurred in the past. Descriptions of the sublines of 402A and 402C are presented according to the pattern of differentiation they exhibit.

Each kind of tissue represented in the primary tumors, as reported by Stevens and Hummel (*2*), was observed in transplantable sublines of 402. Notochord was observed frequently in certain sublines but not as often as in the primary tumors. Bone marrow, hair follicles, adipose tissue, and ganglion cells were only rarely encountered. Thyroid tissue, which was not observed in the primary tumors, was present in a recently observed graft of subline 402A VI.

Structures resembling mouse embryos of about 7 days of age are often seen in grafts of the transplantable sublines of 402. These formations are composed of ellipsoidal vesicles of epithelium like embyronic ectoderm. A single layer of cells reminiscent of embryonic endoderm may envelop the ectoderm, and mesenchymal cells resembling embryonic mesoderm may occupy an intermediate position. These embryoid bodies have not been observed in primary strain 129 teratomas, even in those found in very young mice—to be described in a future publication.

1) *Growths composed entirely of undifferentiated cells.*—Even though none of the sublines of 402 has lost the capacity to produce differentiated tissues, this category is included because of the following facts: *a*) Some of the sublines discussed under category 2 are made up largely of embryonal cells and seem to be evolving in this direction, and *b*) transplantable teratoma 233 is composed exclusively of embryonal cells (fig. 2) that have apparently lost their capacity to differentiate. The embryonal cells are large and show many mitotic figures. The cytoplasm is clear and the nuclei stain heavily with hematoxylin. They resemble cells in 6- or 7-day mouse embryos.

2) *Growths composed predominantly of embryonal cells and immature tissues.*—A branch of subline 402A II (*see* text-fig. 1) is composed of undifferentiated embryonal cells as well as neural tissue, various types of epithelium, cartilage, and bone. In the samples observed histologically, the proportion of the tumor composed of embryonal cells fluctuated somewhat from generation to generation.

Undifferentiated embryonal cells became increasingly predominant in a branch of subline 402A III. The proportions of the tumor composed of undifferentiated cells rose gradually from approximately 5 percent at the 2nd transplant generation to approximately 85 percent in the 37th generation. In addition, cartilage, epithelium, and neural tissue were present. Another branch of 402A III was started at the 11th generation and at the 37th transplant generation approximately 85 percent of the tumor was composed of embryonal cells and immature neuroepithelium.

A branch of 402A I resembled the transplantable tumors described here but was not followed beyond the 10th generation.

3) *Growths composed principally of neural tissue.*—Subline XI was composed mainly of immature and adult neural tissue (fig. 3) for 4 generations and ceased to grow progressively in the 10th transplant generation.

4) *Growths composed predominantly of embryonal cells, cartilage, and muscle.*—Subline 402A X is maintained as 5 parallel branches which have been separated from one another for 25 to 40 transplant generations. All existing sublines are like one another in that cartilage, muscle, and embryonal cells are conspicuous components, while epithelial structures are uncommon (fig. 4). In addition, these branches of 402A X are alike in growing faster than other sublines of 402A. The amount of cartilage in the sections examined varied from approximately 5 to 50 percent. The sample examined histologically from the progenitor of subline X contained no cartilage. In all succeeding generations, from the 3rd to the 51st, cartilage was present and represented a prominent component of this tumor in most cases. There is no indication that the branches of subline 402A X have changed in their capacity to produce cartilage and muscle. Grafts of cartilaginous and noncartilaginous portions of tumors have been deliberately selected in an attempt to establish sublines differing in their chondrogenic capacities. Although attempts to date have failed, they are being continued.

A branch of 402C was separated at the 2nd transplant generation. All 22 samples examined histologically contained cartilage and 17 were considered to have cartilage in amounts over what is usual for transplantable tumors derived from 402, with the exception of subline X.

5) *Growths composed predominantly of well-differentiated tissues of several types.*—Most of the samples of subline 402A VI prepared for microscopic observation show a high degree of differentiation of many tissue types and a relatively small proportion of embryonal cells (figs. 5, 6, 7, 8, and 9). All existing branches of this tumor are descended from fragments that were cultivated *in vitro* for 113 days and then regrafted into mice. Nine grafts of branches transplanted for many (12–22) generations differentiated into adult-type tissues and failed to grow progressively (*see* text-fig. 1). It should be emphasized that these grafts were recovered as living tissues many months after transplantation, which suggests that failure to grow was not due to histo-incompatibility. They apparently lost attributes of progressive growth and acquired qualities of normal tissues. Muscular and ciliary movements, fat accumulation, mucinous and thyroid-colloidal secretion, and blood-cell formation attest to the functional capacities of these tissues. The intervals between successive transplant generations of subline 402A VI varied from 2 weeks to several months, and, as a general rule, the slower growing grafts with longer transplant intervals contained a greater diversity of tissue types. It is necessary to maintain several parallel branches of subline 402A VI so that it will not be lost as a transplantable tumor.

6) *Growths that differentiate completely and fail to grow progressively.*—As mentioned, some of the grafts of 402A VI differentiated into adult-type tissues and ceased to grow progressively. Sublines 402A IV, V, VII, XII, XIII, and XIV also failed to grow in the 2nd to the 7th transplant generations. In addition, branches of sublines I and III differentiated fully and were not transplantable. Samples of these grafts prepared for histological examination were devoid of embryonal and immature elements and were composed of epithelial cysts, nodules of bone and

cartilage, and other adult tissues. The tumors from which these grafts were derived were slow-growing; they were regrafted at an average interval of approximately 45 days up to the time they ceased to proliferate.

7) *Growths composed entirely of an unrecognized tissue.*—A branch of 402A II was homogenized and injected intraperitoneally at the 4th transplant generation. Ascitic fluid, which accumulated following this injection, was inoculated subcutaneously, and succeeding generations were maintained by mincing portions of solid subcutaneous growths and grafting by trocar. The descendant grafts contained areas of unrecognized cells resembling trophoblast (fig. 10). The cells are irregular to cuboidal in shape, with large, oval nuclei. They may be arranged in groups outlined by flattened endothelial-like cells or they may assume an epithelial-like arrangement. There is a conspicuous, homogeneous eosinophilic material between cells or groups of cells. Mitotic figures are quite numerous.

During the 4 transplant generations following the intraperitoneal inoculation of homogeni ed tumor, the proportion of cells resembling mouse trophoblast increased steadily from approximately 10 to 100 percent. This change from a tumor of mixed structure to a simple type has persisted to the most recently observed sample (over 30 transplant generations). Other branches of subline 402A II have evolved in a similar manner.

A branch of subline 402A IX became completely trophoblast-like at the 14th transplant generation and was discarded at the 16th. Prior to the 14th transplant generation approximately 30 to 40 percent of the tumor was composed of immature neural tissue, but this component abruptly disappeared. Subline 402A XV in the 3rd generation was composed mainly of neural tissue with little epithelium. The 4th transplant generation and all subsequent generations to the 11th, when this subline was discontinued, were comprised of trophoblast-like cells. Sublines IX and XV were never homogenized and injected intraperitoneally.

Growth Rates of Sublines of 402

The data presented in table 3 indicate that differences exist in the rates of growth among some of the sublines derived from the primary teratoma 402. Female hosts 6 to 8 weeks of age were inoculated subcutaneously by trocar with minced grafts of each of 5 different sublines. An attempt was made to use approximately the same amount of tissue for each graft. Three weeks later the hosts were sacrificed and the tumors excised and weighed. Subline 402A X, which is chondrogenic, reached a greater weight during the 3-week period than the highly differentiated subline VI.

TABLE 3.—*Wet weights of grafts of 5 sublines of 402—21 days after transplantation**

Transplant generation	Subline	Number of grafts	Mean weight (*gm.*)	Range
44	402A X	9	7. 2	4. 1–12. 3
16	402A VI	9	0. 2	0. 1– 0. 4
36	402A III	9	1. 3	0. 6– 2. 2
39	402A II	9	1. 6	0. 5– 2. 9
37	402C	9	1. 2	0. 3– 4. 1

*All grafts were weighed during the same period of time.

The other sublines fell in-between these extremes. Subline 402A X usually reaches transplantable size in 18 (14 to 25) days, while subline 402A VI usually requires 32 (26 to 51) days. As shown in text-figure 1, the number of transplant generations to date for subline 402A X is greater and the number of generations for subline VI is relatively smaller than for other sublines of 402.

Discussion

It is generally accepted that teratomas in mice owe their capacity for progressive growth to the presence of pluripotent embryonal cells (*1*, *2*, *5*, *6*). Previous studies have shown that embryonal cells are less frequently found in strain 129 teratomas of older animals and that progressive growth in them usually ceases (*2*). Most grafts of primary strain 129 teratomas survive indefinitely as nodules of adult-type, nonproliferating tissues. Four of 19 donors were less than 20 days of age, a time when a large proportion of strain 129 teratomas contain embryonal cells, but none of these 4 yielded progressively growing transplantable tumors. It is presumed that the immature components in them failed to proliferate and differentiated into non-neoplastic tissue.

Foulds (*7*, *8*) has advanced the general proposition ". . . that the structure and behavior of tumors are determined by numerous 'unit characters' which within wide limits are independently variable and capable of independent progression and which can be assorted and combined in a variety of ways." He also states, "Progression occurs frequently in transplanted tumors probably because transplantation prolongs indefinitely the time and opportunities available for it and also, by selective action, favors the predominance of the changed cells which, at first, may constitute a small fraction of the whole tumor." By using morphological criteria, some of the evolutionary fates exhibited by transplantable strain 129 teratomas have been classified into 7 histogenetic categories ranging in complexity from monocellular to highly mixed growths. Some of these types of growths, such as those composed entirely of embryonic or trophoblast-like cells, probably reflect irreversible changes. Other types, however, are probably still capable of progression toward undifferentiated, trophoblast-like, or entirely differentiated tissues exhibiting cessation of growth. An example of this kind of behavior was shown by a branch of subline 402A II. In the 2nd transplant generation 20 percent was composed of embryonal cells and immature neuroepithelium; this gradually changed, becoming 95 percent by the 38th generation. Similarly in subline 402A III, the proportion of embryonal tissue increased from approximately 5 to 85 percent in 37 generations. A change in the opposite direction toward decreasing proportions of embryonal cells and increasing proportions of fully differentiated tissues is illustrated by the behavior of subline 402A VI. The relative amounts of various tissue types in some sublines change from generation to generation, and both gradual and abrupt changes have been observed. In several cases, grafts have lost their embryonal nature and have become completely differentiated.

Probably most, if not all, of the tissues in these teratomas originate from a pluripotent stem cell, and differences between sublines reflect differences in the developmental capacities of their stem cells. This is indicated by the fact that the pleomorphic subline 402A VI, and others, consistently produces growths of mixed tissues in which specific tissue types do not overgrow and supersede others. The predominantly cartilaginous nature of subline 402A X is regarded as being inherent in the stem cell which, even though pluripotent, differentiates more readily into cartilage than into other tissue types. A similar explanation is offered for mouse teratomas that are primarily neurogenic. We do not believe that the neurogenic or chondrogenic components have been "sorted out" in the sense used by Fekete *et al.* (*6*) in attempts to sort out pigmented and white areas by selecting their graft material. Several unsuccessful attempts were made to establish cartilaginous sublines from 402C and 402A VI by grafting material rich in cartilage, and attempts were also made to select noncartilaginous areas for transplantation of subline 402A X in an effort to eliminate cartilage, again without success. If all the tissues share in the malignant qualities of teratomas as Willis (*9*) has suggested, it would seem possible to select grossly identifiable tissues for grafts during the course of many transplant generations and establish them as tumors free, or relatively free, of other tissue types. This has not been found to be true.

We do not ascribe the differentiation of morphologically distinct sublines of 402 to specific environmental influences such as those observed by Hilberg (*10*) for the behavior of an anaplastic osteogenic sarcoma in the mouse, which underwent morphological alterations according to the site of growth. All the subline differences of transplantable strain 129 teratomas have resulted from means beyond our control.

The tissues observed in strain 129 transplantable teratomas include most of those in the primary growths in animals 20 to 30 days of age (*2*). The following types of tissues were commonly found in both the transplantable tumors and primary growths: embryonal cells; nervous tissue; various kinds of epithelia lining cysts, tubules, and glandular formations; cartilage; bone; skeletal, cardiac and smooth muscle; and notochord. Hair follicles, bone marrow, adipose tissue, and ganglion cells were only rarely observed. In addition to the kinds of tissues observed in the primary tumors, thyroid gland was seen in a recent graft of subline 402A VI.

Neural tissue was the most common differentiated component in both the primary and transplantable tumors and it occurred when other kinds of tissues were inconspicuous or missing. Holtfreter (*11*, *12*) pointed out that a great variety of artificial stimuli can elicit neural differentiation in amphibian gastrula ectoderm by exerting a subcytolytic effect. We have no evidence linking cellular injury with neural differentiation in strain 129 teratomas; however, the ubiquity of neural tissue, compared with other tissue types, indicates that in these tumors neuralization is initiated by a comparatively nonspecific stimulus. This interpretation

is in accord with the opinion expressed by Rose (*13*) in a discussion of cellular interaction during embryonic differentiation. He stated that ". . . throughout the period when investigators sought specific chemical inductors, whenever treated ectoderm became neural tissue it was assumed that the treatment had directed ectoderm towards neural differentiation. Instead, it now appears that neural tissue is what unspecifically activated ectoderm becomes. It seems to be the first to appear of a series of ectodermal types."

In some respects the transplantable strain 129 teratomas resemble primary and metastatic teratomas in the human being. According to Willis (*14*) metastases from teratomas may be composed of a single tissue component which may be carcinomatous or sarcomatous, of complex or mixed structure, or may be chorioepithelioma-like. The tendency of cartilage to overgrow other elements has long been recognized (*15*). As revealed by the cartilaginous nature of subline 402A X, strain 129 teratomas are susceptible to a similar change. Neuroblastic metastases of human testicular teratomas have been reported (*16*), and some grafts of strain 129 transplantable teratomas were composed principally of immature and adult neural tissue. It is possible that the unrecognized tissue in the transplantable tumors may be homologous with the trophoblastic elements that Friedman and Moore (*16*) observed in many human testicular tumors. These investigators pointed out the trophoblastic potencies of teratoid and carcinomatous tumors, and reported that metastases of embryonal carcinomas, teratocarcinomas, and teratomas frequently contain chorioepitheliomatous tissue.

Willis (*14*) cited a remarkable group of human cases in which small primary growths of testicular teratomas consisted only of well-differentiated, benign-looking tissues, yet fatal, rapidly growing metastases developed. He presented as the only feasible explanation the concept that the testis must originally have contained a small but actively growing teratoma which produced actively growing metastases, while the residue of the primary growth in the testis underwent maturation to fully differentiated quiescent tissues. Friedman and Moore (*16*) have also offered this explanation. A comparable situation where actively growing tumor becomes quiescent through differentiation was occasionally observed in strain 129 transplantable teratomas, as illustrated by the behavior of some grafts of subline 402A VI. In several instances, grafts of this subline have survived but failed to grow progressively after having flourished for as many as 22 transplant generations—a period of over 3 years—as a transplantable tumor.

Concerning the mechanisms involved in changes in the biological properties of transplantable tumors, G. Klein (*17*) and E. Klein (*18*) have suggested that a mutation-selection system may play an important role in the evolution of some transplantable mouse tumors from a solid to an ascites form. Foulds (*8*) has suggested that the basic idea of progression may be the same as that of epigenetic embryological development. He stated that " . . . there is no more evidence for a persisting 'carrier' of

progression than for a persisting carrier of embryonic development and no greater or less reason to expect one."

We are unable to contribute toward an explanation of the mechanisms involved in the transformation of the stem cells of transplantable strain 129 teratomas at this time. However, certain observations may be mentioned. First, the morphological features characterizing the sublines of 402 are heritable; some have "bred true" for many transplant generations. Second, some of the changes appear to be irreversible. Third, the changes have usually been in the direction of decreasing pluripotency, the stem cells becoming restricted in their capacity for differentiation into many tissue types. Some changes of this type might be compared with the restriction of potency or determination which occurs during embryonic development. The changes from highly mixed growths to growths composed predominantly of nervous tissue, cartilage, or trophoblast may be considered as examples of stem cells that have become determined. The possibility that cytoplasmic alterations are responsible for the differences established between the sublines should be considered. A mutation-selection system, such as that suggested by G. Klein (*17*) and E. Klein (*18*), could account for the differences in the stem cells of the various sublines derived from the primary teratoma 402. We intend to continue to investigate the factors involved in morphological changes in transplantable tumors with strain 129 teratomas. Experimental embryologists might find transplantable teratomas a useful tool in the study of factors involved in mammalian-tissue differentiation.

Summary

Grafts of primary testicular teratomas of strain 129 mice may grow progressively and survive serial transplantation indefinitely, or they may differentiate completely into adult-type tissues and cease to proliferate. The variety of tissues found in strain 129 teratomas are all derived from embryonal cells, and these cells are necessary to insure continued proliferation of the graft. Progressively growing transplantable tumors have been observed, for over 3 years, for changes in the kinds of tissues produced. By grafting pieces of these tumors into large numbers of recipients, ample opportunity was given for the originally highly mixed teratomas to exhibit changes. The kinds of growths evolving during serial transfer were classified as follows: 1) growths composed entirely of undifferentiated cells, 2) growths composed predominantly of embryonal cells and immature tissues, 3) growths composed principally of neural tissue, 4) growths composed predominantly of embryonal cells, cartilage, and muscle, 5) growths composed predominantly of well-differentiated tissues of several types, 6) growths that differentiated completely and failed to grow progressively, and 7) growths composed entirely of an unrecognized tissue.

Most of the tumors described here are from sublines derived from a single primary testicular teratoma designated 402. Some of the sub-

lines became morphologically different from others and the kinds of growths observed included all of those listed except the first.

Growths of types 1, 6, and 7 are considered as reflecting irreversible changes. Tumors in the other categories have remained remarkably stable; however they are considered to be labile and susceptible to change.

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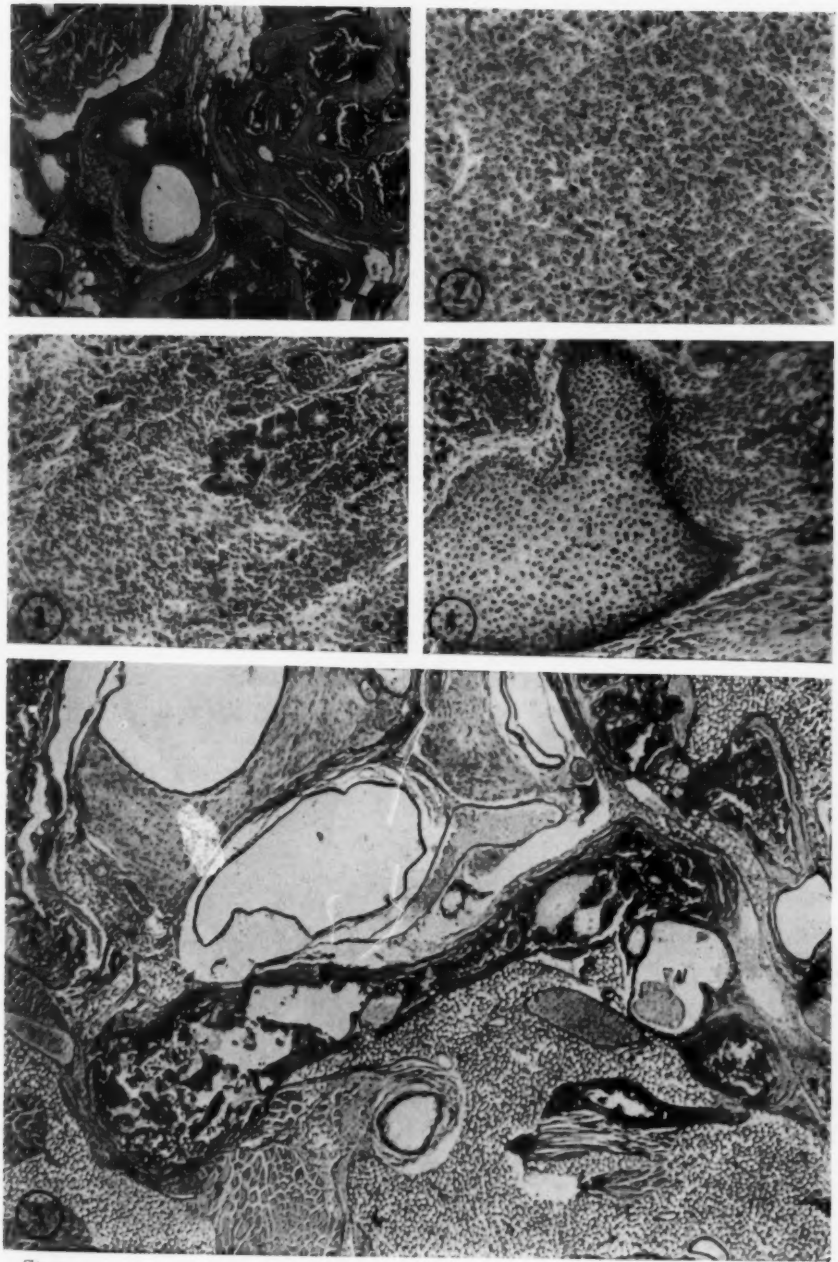
PLATES

463225—58——26



Stevens

463225—58——27

PLATE 124

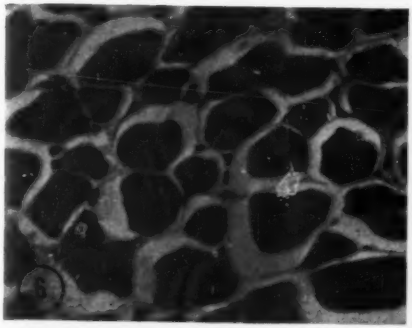
FIGURE 6.—Cross section of skeletal muscle fibers in a graft of subline 402A VI. × 375

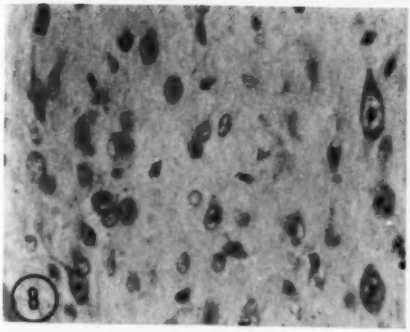
FIGURE 7.—Mixed gland with serous and mucous cells adjacent to muscle fibers and hyaline cartilage. × 375

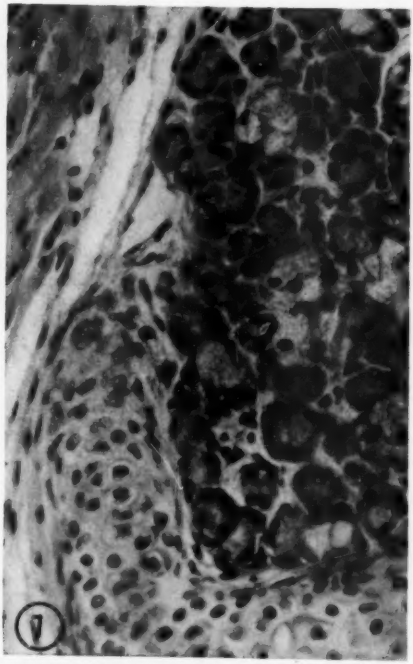
FIGURE 8.—Nervous tissue in graft of subline 402A VI. × 375

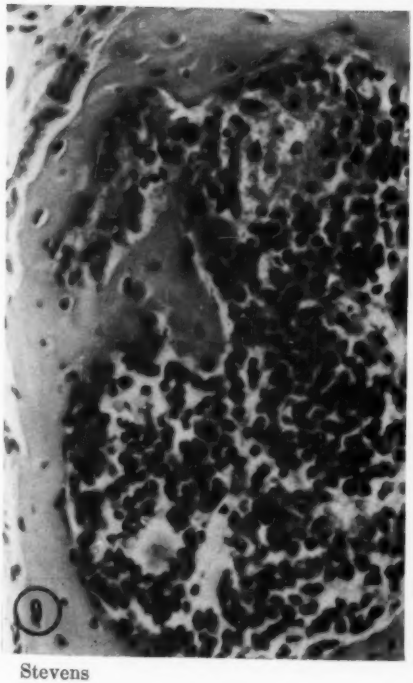
FIGURE 9.—Bone with marrow in graft of subline 402A VI. × 375

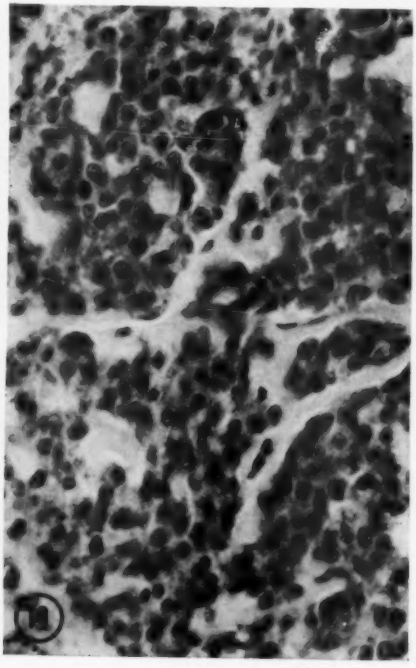
FIGURE 10.—Cells that resemble mouse trophoblast in graft of a branch of 402A II. *Note* homogeneous material between groups of cells. × 375











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Elvin A. Kabat
Nathan Kaliss
Henry S. Kaplan
Fred Karush

Lola S. Kelly
Lawrence Kilham
Arthur Kirschbaum
George Klein
Masuo Kodani
Henry I. Kohn
Peo C. Koller
Bert N. La Du
Maurice Landy
Lloyd Law
Joseph Leighton
Albert Levan
Leon Levintow
Ching C. Li
Arthur J. Lindsey
J. F. Loutit
Ross C. MacCardle
Brian MacMahon
T. Makinodan
Richard A. Malmgren
Nathan Mantel
Clement L. Markert
Paul A. Marks
S. P. Masouredis
Alton Meister
Donald J. Merchant
Ruth Merwin
Florence K. Millar
James Miller
Robert W. Miller
Carl V. Moore
J. F. Morgan
Lincoln Moses
Eli M. Nadel
Alex B. Novikoff
S. Ohno
C. P. Oliver
Donald M. Pace
Harvey M. Patt
A. G. E. Pearse
N. Petrakis
Frederick S. Philips
Michael Potter
Richmond T. Prehn
Alfred M. Prince
Elizabeth S. Russell
Avery A. Sandberg
Katherine K. Sanford
Frank M. Schabel, Jr.
Robert O. Scow
S. H. Seal
Bernard Shacter
Murray J. Shear
Philippe Shubik
Eric L. Simmons
Howard E. Skipper
Stephen S. Sternberg
C. Chester Stock
Robert E. Stowell
Robert L. Straube
Robert J. Taylor
J. H. Tjio
John J. Trentin
Delta E. Uphoff
Aage Videbak
E. R. Vollmer
Charity Waymouth
Sidney Weinhouse
Benton B. Westfall
Richard W. Whitehead
J. Walter Wilson
Seymour H. Wollman
Charles M. Woolf
George W. Woolley
George Wright
Julius S. Youngner

INDEX OF AUTHORS

Volume 20

January 1958 • June 1958

Journal of the National Cancer Institute

Page numbers in italics indicate senior authorship

Adams, Mabelle E., 741
Albert, Mary Day, *309*, *321*
Algire, Glenn H., *1187*
Anderson, Elizabeth P., 53
Andervont, Howard B., 409, *431*
Arostegui, Gonzalo E., 665

Bang, F. B., *329*
Barnes, Glover W., 1123
Beard, J. W., 351
Beaudreau, G. S., *339*, *351*
Becker, Caroline, 339, 351
Bern, Howard A., 541
Bernstein, Seldon E., *577*
Bird, Harris H., 1141
Borders, Mary L., 1187
Borgese, Ninette, 1223
Borsos, Tibor, *1215*
Bryant, Bradley F., 691
Burmester, B. R., 1245
Burstone, M. S., *601*

Caldwell, B. C., 851
Chu, Ernest H. Y., *383*
Colter, John S., *1141*
Condit, Paul T., 71
Cook, James E., *905*
Coulter, Elizabeth Jackson, 79
Crile, George, Jr., *229*

Dalton, A. J., 633
Davidson, E., 1011
Day, Eugene D., *1123*
Defendi, Vittorio, 1141
Del Vecchio, Pasco R., 1203
DeOme, Kenneth B., 541
duBuy, H. G., *403*

Earle, Wilton R., 121, 775
Ebina, Toshiaki, *1023*
Eddy, Bernice E., *747*, 1223
Eichwald, E. J., *563*
Evans, Virginia J., 775, 1187

Feldman, Michael, *513*
Ferguson, S. A., 1011
Fioramonti, Mary C., 121, 775
Fitzgerald, D. B., *763*
Flippin, Reid S., 495
Friedman, Irving A., 1037
Fritz, Richard D., 53

Gardiner, Rita C., 71
Gengozian, Nazareth, 591
Giles, Norman H., 383
Glenner, George G., 63
Goldin, Abraham, 211
Goldstein, Milton N., *675*
Griesemer, Richard A., *949*
Grobstein, Clifford, *107*

Habermann, Robert T., *933*, *979*
Haguenau, Françoise, *633*
Haley, R., 329
Hamilton, James B., *409*
Hartwell, J. L., 763
Heller, Emmanuel, 555
Hesselbach, M. L., 403
Hobbs, Gwendolyn L., 121
Hollander, Melvin, 409
Hueper, W. C., 37
Humphreys, Stewart R., *211*

Ilbery, P. L. T., *1051*

463225—58——28

Kaliss, Nathan, *691*
Kampschmidt, Ralph F., *741*
Kelly, Margaret G., *1113*
Kilham, Lawrence, *729*
Koller, P. C., 1051
Kuratsune, Masanori, *37*
Kurosu, Moriji, 1023

Lampkin, Julia McCain, *1091*
Landau, Bernard R., 53
Law, L. W., *489*, 617
Leiter, J., 763, 1113
Leithold, Stanley L., 1037
Levine, Herbert J., *63*
Levy, Allan H., *53*
Little, Clarence C., *441*
Livingston, W. Steele, *245*
Llambes, Juan L., 665
Lombard, Louise S., 1
Loutit, J. F., 1051

McCoy, Thomas A., 741
McQuilken, William T., 775
Main, Joan M., 207
Makino, Sajiro, *465*
Makinodan, Takashi, *591*
Malmgren, Richard A., *417*, *1203*
Mancuso, Thomas F., *79*
Mantel, Nathan, 211
Maurer, Fred D., *867*, *871*
Merwin, Ruth M., 121
Mickelson, M. N., *495*
Mider, G. Burroughs, 747
Moloney, J. B., 633
Morris, Harold P., *1*
Murray, J. E., *189*
Mustacchi, Piero, *825*

Navarrette, Antonio, 665
Nelson, John B., *911*

Odell, T. T., *851*

Painter, J. C., 351
Pappenheimer, Alwin M., *861*, *879*, *921*
Parker, George, 107
Pinkel, Donald, 675
Pitelka, Dorothy R., *541*
Planinsek, Jakob A., 1123
Porter, K. A., *189*
Potter, John F., 1203
Potter, Michael, 489, 1091
Pratt, A. W., *161*, *173*
Prehn, Richmond T., *207*
Pressman, David, 1123
Prince, Alfred M., *147*, *843*
Pruitt, John C., 1203
Putney, F. K., 173

Révész, László, *1157*
Rose, S. Meryl, *653*
Rutman, R. J., 1011

Sachs, Leo, 513, *555*
Sanford, Katherine K., *121*, *775*
Sasaki, Motomichi, 465
Saunders, Leon Z., *875*, *893*, *899*
Schooley, Caroline N., 541
Schrek, Robert, *1037*
Selye, Hans, *581*
Sharp, D. G., 351
Shekarchi, Isabel C., 591
Shimkin, Michael B., 825
Silmser, C. R., 563
Silverman, Milton, *71*
Silvers, Abigail A., 577
Silvers, Willys K., 577
Smith, Norman H., 1113
Snell, George D., *787*
Sokoloff, Leon, *965*
Stevens, Leroy C., *1257*
Stewart, Sarah E., 747, *1223*

Tosteson, T. R., *1011*

Uphoff, Delta E., *617*, *625*

Venditti, John M., 211

Wagner, Billie P., 1
Wallace, Rosyln E., 1141
Walter, W. G., 1245
Waters, Nelson F., *1245*
Watson, Ben E. M., *219*
Weissman, I., 563
Wellings, S. Robert, 541
Williams, Fletcher P., Jr., 933, 979

Wynder, Ernest L., *665*

Yerganian, George, *705*
Yost, Donald H., *957*
Young, Ralph, 747

INDEX OF SUBJECTS

Volume 20

January 1958 • June 1958

Journal of the National Cancer Institute

Adenocarcinoma:
  mammary *see* Mammary tumors
Adenosinetriphosphatase, assay of, in association with virus of avian myeloblastosis, 339
Adrenal glands, excision of, effect on tumor growth, 219
Amaryllidaceae, tumor-damaging activity in, 763
Amethopterin:
  effect of,
    on excretion of *N*-formiminoglutamic acid during therapy with, 71
    on food intake, body weight, tumor growth, and survival time of mice bearing L1210 leukemia, 211
    on glycolysis and respiration of ascites-tumor cells, 1023
Amino acid(s), requirements of tissue cultures of NCTC clone 929 (strain L), 775
Amino acid antagonists, effects on microorganisms and sarcoma 180, 495
Aminopterin, effect of, on glycolysis and respiration of ascites-tumor cells, 1023
Announcements:
  Availability of Copies of Previous Issues, 227
  Endocrine-cancer Relationships, Reprints Wanted, 860
  Gordon Research Conference, 859
  Nomenclature of Cell Strains, 439
  Sixteen-Year Cumulated Index, 227
  Società Italiana di Cancerologia, 228
  Subscriptions, 227
  Survey of Compounds, Supplement 1, 439
  World Congress of Gastroenterology, 651
Antibiotics:
  tetracycline and bone-marrow treatment following X irradiation of rabbits, 189
Antibody(ies) *see also* Immunity studies:
  circulating, in irradiated mice treated with splenic and bone-marrow cells, 591
  effect of, on development of lesions and neutralization of Rous sarcoma virus in embryos, 1225
  radioiodinated, distribution of in rats bearing Murphy lymphosarcoma, 1123
  to Rous sarcoma virus, appearance and disappearance of in chicks, 329
  tumor-localizing, method for purification of, 1123
Antigens *see* Immunity studies
Antimetabolites *see* Amethopterin, Amino acid antagonists (DL-β-aspartyl hydrazine, 4-deoxypyridoxine, L-glutamyl hydrazine, DL-histadine hydrazine), Aminopterin, 8-Azaguanine, 6-Mercaptopurine
Antitumor agent(s) *see* Amaryllidaceae, Amethopterin, Aminopterin, 8-Azaguanine, 3-bis(β-Chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine, Cortisol, Cortisone, 6-Mercaptopurine, 1,9-Di(methanesulfonoxy)nonane, Methyl-bis(β-chloroethyl)amine *N*-oxide hydrochloride, Sarkomycin, Triethylenethiophosphoramide.
Ascites tumor(s) *see also* Ehrlich ascites tumors:
  hepatoma,
    effects of antitumor compounds and irradiation on glycolysis and respiration of cells of, 1023

463225—58——29

Ascites tumor—Continued
lymphoma,
DBA, immunogenetic study of, 513
lymphosarcoma,
6C3HED, immunogenetic study of, 513
sarcoma,
MC1A, immunogenetic study of, 513
Yoshida —— of rat,
ideogram analysis of chromosomes, 465
effects of antitumor compounds and irradiation on glycolysis and respiration of cells of, 1023
Autolysate (tissue) in treatment of spontaneous dog and cat tumors, 245
8-Azaguanine, effect of, on glycolysis and respiration of ascites-tumor cells, 1023
6-Azauracil, effects of, on galactose metabolism in mice, dogs, and man, 53

Bacteria, filtrates, in treatment of dog and cat tumors, 245
Benzo[*a*]pyrene in coffee soots, 37
Bladder, cancer of in Egypt, association with infestation with *Schistosoma hematobium*, 825
Blood, cytologic identification of tumor cells in, 1203
Bone:
——marrow,
homotransplants of, in X-irradiated rabbits, 189
nucleoproteins, human leukemic cells of, antisera against, 1141
protection against X-irradiation injury compared with spleen, 591
protection by in irradiated mice, genetic factors influencing, 617
transplants in rats following sublethal X irradiation, 851
Breeding of Chinese hamster, for use in research, 705

Cancer:
endocrine imbalance in etiology of; speculative review, 229
mortality *see* Statistics
research, biological aspects of, 441
respiratory tract, development of, association of smoking with, in Cuba, 665
theory of self-inhibition in tumors; speculative review, 653
Carbon tetrachloride:
induction of hepatomas in strain C3H mice with, 431
Carbon tetrachloride—Continued
injury, effect of X rays upon mitoses induced by, 309, 321
liver-tumor induction by, in C3H mice, 431
Carcinogenesis *see also* Tumor, specific type:
by irradiation *see* X irradiation
comparison of impaired liver function and morphologic changes in rats fed *N*-2-fluorenyldiacetamide, 1
tumor-producing agent of mice,
carried in tissue culture, production of tumors in mice with, 1223
production of tumors in hamsters by, 747
Carcinogens *see* Benzo[*a*]pyrene, Carbon tetrachloride, *N*-2-Fluorenyldiacetamide, 4-*o*-Tolyazo-*o*-toulidine, Tumor-producing agent, Virus
Cell(s):
cytologic detection of tumor cells in blood, 1203
effect of X-ray-damaged tumor cells on development of admixed viable tumor cells, 1157
Hela, studied in diffusion chambers, 1187
Chemotherapy *see also* Antimetabolites:
amino acid antagonists, singly and combined, effect on tumor and bacterial growth, 496
cortisone-sensitive lymphosarcoma, P1798 response to cortisone and development of resistance in mouse, 1091
Chimaeras, X-radiation-induced, of mouse, immunological characteristics of, 1051
3-bis(β-Chlorethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine (SK 1424), effects of, on glycolysis and respiration of tumor cells, 1023
Chromosome(s):
changes in mouse liver, after X irradiation and carbon tetrachloride injury, 309, 321
comparison of, in Yoshida sarcoma and four sublines, 465
in tissue culture, comparative studies on HeLa and clonal isolates of HeLa, 383
Coffee soots, polycylic hydrocarbons in, 37
Cortisol:
—— acetate, antitumoral effects of in rats, 581

Cortisone:
and adrenalectomy, effects of, on growth rate of ascites tumor, 219
sensitivity to and development of resistance to, by lymphosarcoma P1798, 1091
Cricetulus griseus see Hamster, Chinese
Cytochrome *c* and myoglobin in tumorous rats, 741

1,9-Di(methanesulfonoxy)nonane, effect of on glycolysis and respiration of ascites-tumor cells, 1023
Drug resistance, development of cortisone resistance by lymphosarcoma P1798, 1091

Ehrlich ascites tumor:
effects of,
antitumor compounds and irradiation on glycolysis and respiration of cells of, 1023
cortisone and adrenalectomy in mice on growth rate of, 219
ovarian extracts on lethality of, 1011
Electron microscopy of Rous sarcoma virus, 633
Embryo(s):
chick, natural resistance of, to infection with Rous virus, 843
chick, titration of Rous sarcoma virus on chorio-allantoic membrane of, 147
mouse, metanephrogenic mesenchyme from, tubule formation by at various *in vivo* sites, 107
Environment, role of, in cancer of respiratory tract in Cuba, 665
Epidemiology, relation of cancer of bladder to infestation with *Schistosoma hematobium*, 825
Erythroblastosis (avian) *see* Virus
Estrous cycle of Chinese hamster, 705
Etiology of cancer, association of bladder cancer with *Schistosoma hematobium* infestation, 825
Extract(s):
ovarian, carcinostatic effect on ascites tumor, 1011

Fibroma-myxoma virus transformation *see* Virus
N-2-Fluorenyldiacetamide, impaired liver function and morphologic changes in rats fed, 1
Folic acid deficiency, excretion of *N*-formiminoglutamic acid during, 71

N-Formiminoglutamic acid, method for detection of in urine, 71

Galactose, metabolism, effect on, of 6-azauracil in mice, dogs, and man, 53
Gene(s) *see also* Chromosomes, Genetics:
histocompatability genes,
analysis of in mice, 787
H-2 locus in mouse, relation of, to secondary phase of X-irradiation injury, 617
number involved in skin grafting of mice, 207
Y-linked, attempt to demonstrate in in mouse, 577
Genetics:
factors in mice influencing irradiation protection by bone marrow—*H-2* locus, 617
influence on resistance of chicks to infection with erythroblastosis virus, 1245
Glucose, use by mitochondria in normal and neoplastic mouse tissues, 403
Grafts *see also* Transplantation:
heterografts, in mice, studied in diffusion chambers, 1187
homografts,
factors affecting destruction of, in mice receiving successive tumor inocula, 691
of mouse skin, number of histocompatibility genes involved in, 207
of transplantable tumors in X-radiation-induced mouse chimaeras, 1051
skin,
attempt to demonstrate Y-linked histocompatibility gene by, 577
sex-linked histocompatibility genes in relation to, 555
tumor, sex-linked rejection of, 563
Growth:
of nails of mice carrying the mammary-tumor agent, 409
of transplants of testicular tumors in inbred mice, 1257
of viable tumor cells, influence on of admixed X-ray-damaged tumor cells, 1157
rate of, in Ehrlich tumors, effects of cortisone and adrenalectomy on, 219
regulation of tumor, review of data on, 653
retardation of, in leukemic mice receiving amethopterin, 211

Hamster:
Chinese, history, general care and use of, 705
production of tumors in, by mouse tumor agent passed *in vitro*, 747
Homografts *see* Grafts
Hormones *see also* Cortisol acetate:
endocrine imbalances, role of, in genesis of cancer, 229
Humoral factors, in relation to X-irradiation injury, 309, 321
Hydrocarbons, polycyclic, in coffee soots, 37
17-Hydroxy-11-dehydrocorticosterone: *see* Cortisone

Immunity studies:
antibodies, tumor-localizing, method for purification of, 1123
antisera against human leukemic bone-marrow cell nucleoproteins, cytotoxicity of, *in vitro*, 1141
factors determining homograft destruction and immunological enhancement in mice receiving tumor inocula, 691
influence of antigenic factors in production of antitumor cytotoxic serum, 417
sex-linked histocompatibility antigens, 555
sex-linked rejection of normal and neoplastic tissue in mice, 563
tumor (———)
mechanism responsible for homotransplantability of adapted tumors, 513
relation between development of host immunity and production of sarcomas by two cell lines derived *in vitro* from a single cell, 121
role of histocompatibility genes in, 787
X-radiation-induced mouse chimaeras, immune response in, 1051
Infectious diseases of laboratory rodents, Symposium *see also* Myositis:
Bartonellosis, rodents, 949
Diarrheal diseases, mice, 861
Encephalomyelitis, Theiler's mouse, 871
Encephalitozoon infection, rabbits, 957
JHM virus, mice, 879
Joint diseases, rodents, 965
Lymphocytic choriomeningitis, mice, 867
Mouse pox, infectious ectromelia, 875
Myocarditis and pulmonary arteritis, mice, 921
Infectious diseases etc.—Continued
Parasitic diseases—helminths, rodents, 979
Respiratory infection with pleuropneumonia-like organisms, rats and mice, 911
Salivary-gland virus disease, guinea pigs, 905
Salmonellosis in laboratory animals, 933
Tyzzer's disease, mice, 893
Instrumentation:
respirometer, analytical, for small animals, 161
Iron *see* Metabolism

Kidney, tubule formation, by mouse metanephrogenic mesenchyme transplanted *in vivo*, 107

Lactobacillus delbrueckii, effect of amino acid antagonists on, 496
Leukemia *see also* Lymphoid tumors:
lymphocytic, chronic in man, sensitivity to X irradiation and chemotherapeutic agents *in vitro*, 1037
Liver:
fetal, prevention of secondary irradiation injury by injection of, 625
morphologic changes and liver function of rats fed *N*-2-fluorenyldiacetamide, 1
regenerating, mitotic abnormalities in, following whole and partial-body X irradiation, 309, 321
Liver tumors:
carcinogenesis with in mice
carbon tetrachloride susceptibility to, 431
4-*o*-tolylazo-*o*-toluidine sex difference in susceptibility to, 431
Lymphomatosis *see* Virus—erythroblastosis
Lymphoid (tumors) *see also* Ascites tumors and Leukemia:
carcinogenesis, in mice, by X irradiation, further evidence for indirect tumor induction, 489
leukemia L1210, of mice,
effect of amethopterin on physiology of host bearing, 211
effect of phenazines and related compounds on, 1113
inhibitory effects of 6-azauracil on, 53

Lymphoid—Continued
lymphosarcoma P1798, origin of, resistance and response to cortisone, 1091
Lymphosarcoma *see* Lymphoid (tumors)

Mammary gland(s) *see also* Mammary tumors:
of mouse, virus-like particles in, 541
Mammary tumor(s):
adenocarcinoma C3HBA, effect of phenazines and related compounds on, 1113
MT03 and MT13, transplantation in radiation mouse chimaeras, 1051
Mammary-tumor agent:
increased rate of nail growth in mice carrying, 409
virus-like particles in alveolar nodules of mammary gland of mice infected with, 541
6-Mercaptopurine, glycolysis and effect of on respiration of ascites-tumorcells, 1023
Metabolism:
energy, alterations of, observed in rats receiving Walker tumor transplants, 173
galactose, in mice, dogs, and man, effect of 6-azauracil on, 53
iron, in tumorous rats, 741
of ascites-tumor cells, effects of antitumor compounds on, 1023
Methyl-bis(2-chloroethyl)amine hydrochloride, *in vitro* sensitivity of human leukemic lymphocytes to, 1037
Methyl-bis(β-chloroethyl)amine *N*-oxide hydrochloride (Nitromin), effect of, on glycolysis and respiration of ascites-tumor cells, 1023
Mitochondria, of normal and neoplastic mouse tissues, glucose utilization by, 403
Mitosis, abnormal, in regenerating mouse liver following X irradiation, 309, 321
Mortality *see* Statistics
Myeloblastosis (avian) *see* Virus
Myoglobin and cytochrome *c* in tumorous rats, 741
Myositis in guinea pigs, 899

Neuroblastoma:
cells of (human), long-term growth in tissue culture, 675
Nitrogen mustard *see* methyl-bis(2-chloroethyl)amine hydrochloride, 1037
Nucleic acids *see* Nucleoproteins
Nucleoproteins, of human leukemic bone marrow, as antigens, 1091

Ovarian tumor(s):
teratoma, description of and changes in during prolonged serial transfer, 1257
Ovary, extracts of, effect on lethality of Ehrlich ascites tumor, 1011

Pancreas, glucagon content and tryptophan staining of *alpha* cells of rabbits, 63
Phenazines, and related compounds, relation of structure to activity in mice, 1113
Phosphatase(s):
histochemical demonstraton of, by naphthol AS-phosphates, 601

Plant materials:
tumor-damaging capacity of, Amaryllidaceae, 763

Review:
biological aspects of cancer research, 441
of role of endocrine imbalances in the genesis of certain cancers and degenerative diseases, 229
theory of self-inhibition in tumors, 653
Rose bengal, I^{131} labeled, used as liver-function test, 1
Rous sarcoma *see* Virus—Rous sarcoma

Sarcoma(s) *see also* Ascites tumors; Lymphoid tumors; Rous; Walker carcinosarcoma:
benzopyrene-induced, transplantation in radiation-induced mouse chimaeras, 1051
——producing capacity of two lines of mouse cells derived *in vitro* from 1 cell, 121
——I,
homografts of, in immunity study in mice, 691
transplantation of, in radiation mouse chimaeras, 1051
——37,
antitumor activity of Amaryllidaceae tested against, 763
effect of phenazines and related compounds on, in mice, 1113
——180, effect of amino acid antagonists on, 495

Sarkomycin, effect of, on glycolysis and respiration of ascites-tumor cells, 1023
Schistosoma hematobium, infestation with, association with bladder cancer in Egypt, 825
Sex, differences in rabbit leukocytes, 189
Smoking (tobacco), in Cuba, and environmental factors associated with cancer of respiratory tract, 665
Spleen, cells of, protection against X-radiation injury compared with bone marrow, 591
Stains and staining *see* Techniques—histochemical
Statistics, Cancer
  mortality among native white, foreign-born white, and nonwhite male residents of Ohio, 79
  respiratory-tract cancer, smoking and environmental factors associated with in Cuba, 665
Symposium on some infectious diseases of laboratory rodents, 861–1009

Techniques:
  biochemical,
    ATPase assay, in association with myeloblastosis virus, 339
    detection of *N*-formiminoglutamic acid in human urine, 71
  biological,
    cytologic detection of tumor cells in blood, 1203
    diffusion chamber construction and assembly of, for intraperitoneal implantation, 1187
    double granuloma-pouch technique, antitumor effects of cortisol demonstrated in rats by, 581
  histochemical,
    naphthol AS-phosphates for demonstration of phosphatases, 601
    protein-bound tryptophan, in *alpha* cells of pancreas, 63
  I^{131} rose-bengal as a liver function test, 1
  microbiological, for detection of *N*-formiminoglutamic acid in human urine, 71
Teratoma *see* Ovarian tumors
Tetracycline hydrochloride, reduction in mortality of irradiated rabbits receiving marrow transplants and treatment with, 189
Therapy, tissue autolysate in treatment of dog and cat tumors, 245
Tissue culture:
  chromosomes of HeLa clones, 383
  comparison of sarcoma-producing capacity of two lines derived *in vitro* from single cell, 121
  fibroblast cultures, amino acid requirements of NCTC clone 929 (strain L), 775
  of metanephrogenic mesenchyme from mouse embryo, comparison of effects obtained *in vivo*, 107
  of neuroblastoma (human), long-term growth of, 675
  passage of mouse tumor agent, production of tumors in hamsters following, 747
  release of virus of avian myeloblastosis by myeloblasts in, 351
  selective toxicity of antinucleoprotein antisera, 1141
  transformation of fibroma to myxoma virus in, 729
  tumor-producing agent carried in, induction of tumors in mice with, 1223
4-*o*-Tolylazo-*o*-toluidine, liver tumor induction by, in C3H mice, 431
Toxicity, production of generalized body tremors by phenazinium compounds, 1113
Transplantation:
  alterations in energy metabolism of rats receiving Walker tumor transplants, 173
  of bone-marrow, in sublethally irradiated rats, 851
  homografts, tumor, dosage and growth of in mice, 789
  of homologous bone marrow in X-irradiated rabbits, 189
  of mouse metanephrogenic mesenchyme, formation of tubules by *in vivo*, 107
  serial, of testicular teratomas in inbred mice, 1257
  transplantability of tumors, mechanism of acquired homotransplantation, 513
Triethylene melamine, *in vitro* sensitivity of human leukemic lymphocytes to, 1037
Triethylenethiophosphoramide (thio-TEPA), effect of on glycolysis and respiration of ascites-tumor cells, 1023
Tryptophan, protein-bound, histochemical demonstration of in pancreatic *alpha* cells, 63
Tumor-damaging agents *see* Antitumor agents

Tumor-producing agent,
carried in tissue culture, induction of tumors in mice with, 1223
production of tumors in hamsters by, following passage in tissue culture, 747

Tumors *see also* Names of:
dog and cat, various spontaneous, treatment with placental autolysate, 245
theory of self-inhibition in, speculative review, 653

Virus *see also* Mammary-tumor agent:
avian erythroblastosis,
genetic influence on resistance to induced infection, 1245
avian myeloblastosis,
release of, by myeloblasts in tissue culture, 351
technique for assay of ATPase associated with, 339
Rous sarcoma,
absence of neutralization of anti-normal cell antibodies and complement, 1215
antibodies to, appearance and disappearance of in chicks, 329
electron microscopic and bioassay studies on, 633
mechanism of resistance of chick embryos to infection with, 843
titration of, on chorio-allantoic membrane of chick embryo, 147
transformation of, from fibroma to myxoma in rabbits, 729

Walker carcinosarcoma 256:
alterations in energy metabolism of rats receiving transplants of, 173
cytochrome *c* and myoglobin in rats bearing, 741

X-ray (roentgen) irradiation:
bone-marrow transplants in rats after sublethal doses of, 851
chimaeras induced by, immunological characteristics of, 1051
effect(s) on,
glycolysis and respiration of ascites-tumor cells, 1023
host immune reaction, in relation to sarcoma-producing capacity of cultured cells, 121
leukemic lymphocyte survival *in vitro*, 1037
mitosis in regenerating mouse liver, 309, 321
indirect induction of lymphocytic neoplasms in mice by, 489
injury,
protection against,
genetic factors in secondary phase of irradiation syndrome, 617
preclusion of secondary phase of injury by injection of fetal liver, 625
relative effects of spleen and bone marrow cells, 591
survival of rabbits, following treatment with bone-marrow transplants and antibiotic, 189
tumor cells damaged by, effect on development of admixed viable tumor cells, 1157

Errata

Volume 20, Number 1, January 1958

Page 72, line 3 and line 4: In the paper entitled "A Method for the Determination of *N*-Formiminoglutamic Acid in Urine," by Silverman, *for* "200 μM" *read* "20 μM."

Volume 20, Number 4, April 1958

Page 697, line 16: In the paper entitled "Factors Determining Homograft Destruction and Immunological Enhancement in Mice Receiving Successive Tumor Inocula," by Kaliss and Bryant, *for* "AS/n" *read* "A/Sn."

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The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observctions and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

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Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

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6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. A drawing three times the size of that desired for the final figure is preferable. The lettering should be uniform and of such size that in the reproduction it will be approximately the same size as that of the printed type used in the text. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats, etc.) of each drawing are also submitted, review of the manuscript is expedited.

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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 20 • NO. 6 • JUNE 1958

Page

The carcinostatic effect of extracts prepared from cow ovaries. *T. R. Tosteson, S. A. Ferguson, E. Davidson,* and *R. J. Rutman* ... 1011

Effects of antitumor compounds and radiation on the glycolysis and respiration of ascites-tumor cells. *Toshiaki Ebina* and *Moriji Kurosu* ... 1023

Variability of the *in vitro* sensitivity of human leukemic lymphocytes to X rays and chemotherapeutic agents. *Robert Schrek, Irving A. Friedman,* and *Stanley L. Leithold* ... 1037

Immunological characteristics of radiation chimaeras. *P. L. T. Ilbery, P. C. Koller,* and *J. F. Loutit* ... 1051

Response to cortisone and development of cortisone resistance in a cortisone-sensitive lymphosarcoma of the mouse. *Julia McCain Lampkin* and *Michael Potter* ... 1091

Relation of chemical structure to development of tumor damage and body tremors in a series of phenazines and related compounds. *Margaret G. Kelly, Norman H. Smith,* and *Joseph Leiter* ... 1113

Improved methods for the purification of tumor-localizing antibodies. *Eugene D. Day, Glover W. Barnes, Jakob A. Planinsek,* and *David Pressman* ... 1123

Immunological studies with nucleoproteins from tissue-culture cells. I. Nucleoproteins from human leukemic bone-marrow (MCN) cells. *John S. Colter, Vittorio Defendi, Roslyn E. Wallace,* and *Harris H. Bird* ... 1141

Effect of lethally damaged tumor cells upon the development of admixed viable cells. *László Révész* ... 1157

Studies of heterografts in diffusion chambers in mice. *Glenn H. Algire, Mary L. Borders,* and *Virginia J. Evans* ... 1187

A method for the cytologic detection of tumor cells in whole blood. *Richard A. Malmgren, John C. Pruitt, Pasco R. Del Vecchio,* and *John F. Potter* ... 1203

Absence of neutralization of Rous sarcoma virus by antinormal-chicken-embryo serum and complement. *Tibor Borsos* ... 1215

Neoplasms in mice inoculated with a tumor agent carried in tissue culture. *Sarah E. Stewart, Bernice E. Eddy,* and *Ninette Borgese* ... 1223

Genetics of experimentally induced erythroblastosis in chickens. *Nelson F. Waters, B. R. Burmester,* and *W. G. Walter* ... 1245

Studies on transplantable testicular teratomas of strain 129 mice. *Leroy C. Stevens* ... 1257

Acknowledgment ... 1277

Index of authors ... 1279

Index of subjects ... 1281

6784